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EDITORIAL

Postoperative ileus: Impact of pharmacological treatment, laparoscopic surgery and enhanced recovery pathways

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Abstract

Almost all patients develop postoperative ileus (POI) after abdominal surgery. POI represents the single largest factor influencing length of stay (LOS) after bowel resection, and has great implications for patients and resource utilization in health care. New methods to treat and decrease the length of POI are therefore of great importance. During the past decade, a substantial amount of research has been performed evaluating POI, and great progress has been made in our understanding and treatment of POI. Laparoscopic procedures, enhanced recovery pathways and pharmacologic treatment have been introduced. Each factor has substantially contributed to decreasing the length of POI and thus LOS after bowel resection. This editorial outlines resource utilization of POI, normal physiology of gut motility and pathogenesis of POI. Pharmacological treatment, fast track protocols and laparoscopic surgery can each have significant impact on pathways causing POI. The optimal integration of these treatment options continues to be assessed in prospective studies.

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Key words: Postoperative ileus; Pathophysiology; Cost utilization; Pharmacologic treatment; Laparoscopic surgery; Enhanced recovery pathways

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INTRODUCTION

All patients with a bowel resection develop postoperative ileus (POI), an interruption of bowel function after surgery[1-4]. POI is characterized by a transient cessation of bowel function with a variable reduction in motility sufficient to prevent effective transit of intestinal contents[5]. POI is the single most important determinant of length of stay (LOS) after abdominal surgery, and thus has significant implications for individual patients and hospital resource utilization.

POI has been discussed by surgeons for more than two centuries[6,7]. In 1906, Finney divided POI into three subgroups according to pathophysiology: mechanical, septic and adynamic[8]. After a century of debate a conse-

nsus conference in 2006[5] proposed a definition of POI as: "transient cessation of coordinated bowel motility after surgical intervention, which prevents effective transit of intestinal contents or tolerance of oral intake". Primary POI was defined as such cessation occurring in the absence of any precipitating complication, whereas secondary POI was defined as that occurring in the presence of a precipitating complication (infection, anastomotic leak, *etc*.). Patients undergoing major abdominal surgery are at highest risk for developing POI, which is related to the degree and length of manipulation of the intestines. Other surgical procedures may also be associated with POI, such as cardiac surgery, orthopedic surgery and trauma[9-12]. In addition to surgery and trauma, postoperative opioid analgesics that are necessary to manage postoperative pain contribute significantly to the incidence of POI.

Clinically POI is characterized by the inability to tolerate a solid diet, delayed passage of flatus and formed stool, pain and abdominal distention, nausea, vomiting, and accumulation of gas or fluids in the bowel[9,13]. Several pharmacological substances have been introduced to treat or prevent POI[14-26]. There has however been little if any proven success in pharmacologic limitation of POI until the reports investigating selective antagonism of opioid receptors.

In addition to pharmacological treatment, fast track or enhanced recovery pathways (ERP) and laparoscopic surgery have introduced new dimensions in treatment of POI. ERP shorten the postoperative recovery period[27] and laparoscopic surgery shortens the average length of POI[28]. The objective of the present paper is to highlight POI and its consequences for patients and society, and the impact pharmacological treatment, laparoscopic surgery and ERP have upon the pathogenesis and duration of POI.

COST IMPLICATIONS OF POI

POI as a complication of major abdominal surgery can have a substantial clinical and economic impact. POI is associated with increased postoperative morbidity, reduced patient satisfaction, and increased length of hospital stay. Moreover, POI-related increases in LOS and use of resources translated into increased costs for the health care system[29]. It has been estimated that POI accounts for a significant amount of perioperative health care costs in the US[5], with estimated total hospital costs attributable to POI $1.28 billion[5].

A recently published study by Iyer *et al*[30] identified 17 000 patients undergoing colectomy in the US Premier Perspective database. The mean hospital LOS was significantly longer in patients with POI compared with patients without POI (13.8 d *vs* 8.9 d). POI in colectomy patients is a significant predictor of increased hospital resource utilization.

Clearly, reducing the LOS associated with POI has become a health care priority for the surgical community, with interventions reducing the LOS having great implications both for the individual patient and for hospital cost utilization.

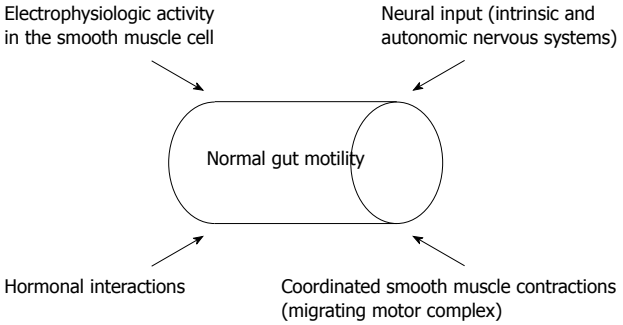


Figure 1 Normal physiology of gut motility.

Bell *et al*[31] recently published a paper, where the economic effect of the use of alvimopan in four randomized controlled trials (RCT) trials was analyzed. This paper used LOS data from the North American alvimopan trials, and calculated cost by extrapolation of cost from the Premier Perspective database, and cost of medication, using mathematical modeling techniques. Compared with placebo, alvimopan was associated with a significantly shorter mean time to gastrointestinal (GI) recovery and a mean hospital LOS of one full day less than placebo. The mean estimated hospital cost was $879-$977 less for patients who received alvimopan compared with placebo. Bell *et al*[31] suggest that use of alvimopan compared with placebo may have a cost-saving effect in the hospital setting. It is however unresolved if alvimopan is more cost effective than optimal use of laparoscopic surgery and ERP protocols, and further research is needed.

PATHOGENESIS OF POI

Normal bowel function is a complex interaction between GI motility, mucosal transport and defecation reflexes. The motility of the intestines is dependent upon the electrophysiological activity in the smooth muscle cells, neural input from the intrinsic and autonomic nervous system, hormonal interactions and coordinated smooth muscle interaction[32] (Figure 1). The migrating motor complex (MMC) has a central position in the normal gut motility[10,33]. The MMC is divided into four phases and regulates the gut motility (contractile pattern) between meals[34] and occurs approximately once every 1-2 h; Phase Ⅰ: Oscillating smooth muscle membrane potentials without actual muscle contractions. Phase Ⅱ: Occurrence of intermittent muscle contractions. Phase Ⅲ: Contractions increase to the maximal contractile frequency (i.e. stomach 3 contractions/min and duodenum 11 contractions/min). Phase Ⅳ: cessation of contractions, and the bowel returns to Phase Ⅰ.

In addition, the nervous system (enteric and central)[35], hormones[36] and smooth muscle activity play roles. Ingestion of food activates these mechanisms and turns off the MMC contractile pattern.

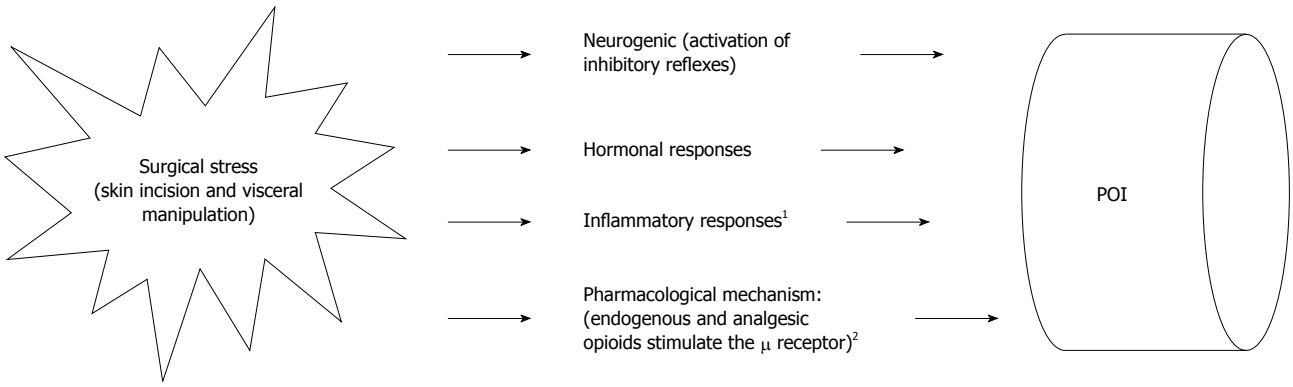


Figure 2 Pathogenesis of postoperative ileus. [1]Laparoscopic surgery decreases the surgical stress and inflammatory response; [2]Primarily exogenous opioids, e.g. morphine, binding to μ-receptors in the GI tract, which results in disorganized and non propulsive motility and, thus, prolongs ileus. In addition, activation of opioid receptors, which occurs following major abdominal surgery, inhibits acetylcholine release, reduces gastrointestinal motility, and has been demonstrated to play a key role in POI regulatory pathways[10,29,36-38]. Alvimopan inhibits this effect by blocking the pheripheral opioid μ receptor.

The interactions between these regulatory components are complex and much is unknown. Contractions in the colon differ from the small intestine, with irregular oscillations and contractile patterns[10,35].

Although multiple factors are thought to contribute to the pathogenesis of POI, four major pathways have been identified (Figure 2): (1) Neurogenic: surgical stress (i.e. skin incision and bowel manipulation) response stimulates inhibitory neural reflexes resulting in decreased bowel motility. This activation of inhibitory reflexes inhibits the normal MMC pattern and is activated as early as at the moment of skin incision (by somatic fibers) and from manipulation of the intestines (by visceral fibers)[37]; (2) Inflammatory: bowel manipulation and resection stimulates normally inactive macrophages and neutrophil recruitment with release of inflammatory mediators that reduce bowel motility; this includes endogenous opioid peptides. Manipulation also causes secretion of proinflammatory cytokines[37]. An increase in the degree of surgical manipulation leads to increased accumulation of neutrophils, macrophages, mast cells, T cells, natural killer cells and dendritic cells which in turn leads to increased inflammation in the intestine and tissue damage[32]. All these factors induce paralysis of the intestine and in turn POI; (3) Hormonal: surgical stress results in elevation of corticotrophin-releasing factor, which stimulates release of inflammatory mediators in the bowel[38]. In addition, a wide variety of local factors, hormones and neurotransmitters may play a role in POI (i.e. Substance P, Nitric Oxide and Calcitonin gene-related peptide CGRP)[10]; and (4) Pharmacologic: primarily exogenous opioids, e.g. morphine, binding to μ-receptors in the GI tract, which results in disorganized and non-propulsive motility and thus prolongs ileus. In addition, activation of opioid receptors, which occurs following major abdominal surgery, inhibits acetylcholine release, reduces GI motility, and has been demonstrated to play a key role in POI regulatory pathways[10,32,39-41].

Opioid-based regimens are the most common treatments to effectively manage post-surgical pain. However, morphine and other μ-opioid receptor agonists can prolong the duration of POI through delayed gastric emptying, reduced GI motility, and disrupted colonic myoelectric activity[42,43]. In addition to exogenous opioids, studies have suggested a role for endogenous opioid peptides in various postoperative responses, including ileus[43-46].

PHARMACOLOGICAL TREATMENT AND POI

Up to the year 2000, several pharmacological agents had been studied in order to prevent POI, however none of these agents was effective enough to become part of routine established practice[14-26].

Chewing sugarless gum following elective intestinal resection is associated with improved outcomes. Gum chewing is thought to promote physiological simulation of the cephalic-vagal axis, thereby increasing bowel motility and GI stimulation[47-49]. There exist two meta-analyses analyzing the effects of chewing gum[50,51], however, all of the included trials in these meta-analyses were small, and only two trials had an adequate sample size calculation.

The meta analysis showed an improvement in first time to bowel movement of 23 h and a reduction in LOS of 1.1 d, and is similar to the effect shown by alvimopan. An adequately powered, methodologically rigorous trial of gum chewing is however required to confirm if there are any benefits[52].

After five RCT, alvimopan represented a novel breakthrough in pharmacological treatment of POI. Alvimopan is a selective peripherally acting μ-opioid antagonist administered orally, blocking the μ-opioid receptor and minimizing the paralytic effect opiates have upon the intestines. The medication blocks the peripheral μ-receptor with high affinity, therefore minimizing the paralytic effect opiates have upon the intestines (Figure 2). This molecule is large and polar and therefore does not cross the blood brain barrier and therefore does not impair analgesia. During the last 5 years five RCTs have been conducted upon alvimopan and its effect upon POI[53-58]. In all these trials

Table 1 Overview of RCT trials 2004-2008 of alvimopan

Author	Number of patients	Primary end point	GI[2] improvement (h)	GI[3] improvement (h)	Hazard ratio	DCO improvement (h)
Wolff *et al*[57]	510	GI[3]	20.0[1]	15.0[1]	1.28[1]	13.0[1]
			28.0[2]	22.0[2]	1.54[2]	20.0[2]
Viscusi *et al*[56]	666	GI[3]	16.4[1]	7.5[1]	1.20[1]	14.2[1]
			13.7[2]	9.9[2]	1.24[2]	15.2[2]
Delaney *et al*[55]	451	GI[3]	15.2[1]	14.1[1]	1.45[1]	14.0[1]
			10.5[2]	7.5[2]	1.28[2]	7.2[2]
Ludwig *et al*[53]	654	GI[2]	20.0[2]	16.0[2]	1.50[2]	17.0[2]
Buchler *et al*[58]	911	GI[3]	14.3[1]	8.5[1]	1.18[1]	8.1[1]
			10.7[2]	4.8[2]	1.37[2]	5.9[2]

[1]6 mg alvimopan; [2]12 mg alvimopan. Hazard ratio: The ratio of two hazard rates. Hazard rate is defined as the probability to failure given survival to date. DCO: Discharge order; GI[2]: The time to recovery of GI function, a composite end points that represent full upper and lower recovery. i.e. time that the patient first tolerated solid food and time that the patient first passed a bowel movement; GI[3]: The time to recovery of GI function, a composite end points that represents full upper and lower recovery. i.e. time that the patient first tolerates solid food, or time that the patient first passes flatus or a bowel movement.

recovery of bowel function was enhanced, except one from Europe when lower opioid doses were used. The primary endpoints in these trials were time to recovery of GI function, measured as either GI[2] or GI[3], composite measures that were developed to track recovery of upper and lower GI function. In these trials GI[2] was defined as "the time that the patient first tolerated solid food and had passed a bowel movement". GI[3] was defined as "the time that the patient first tolerates solid food, and the patient first passes flatus or a bowel movement".

The results from these trials lead to Food and Drug Administration approval in May 2008. A summary of the trial results is shown in Table 1.

The definition of lower GI recovery has been controversial and debated, and up to date there exists no consensus upon this definition. All of the RCTs chose GI[3] as their primary endpoint for GI recovery, except the study by Ludwig *et al*[53], as GI[2] had been noted to be a more robust endpoint by the collaborative study group. GI[3] uses documentation of passage of flatus which can be subject to considerable variability[59], since the patient has to be conscious and willing to report it. In this way it was advocated that GI[2] is a more objective endpoint[53], i.e. a composite end point that was represented by the time the patient first tolerated solid food and time that the patient first passed a bowel movement. In the trial by Ludwig *et al*[53], GI[2] recovery was primarily driven by time to first bowel movement, as this was the later occurring of the two components of GI[2] recovery. In the RCTs there are no obvious dose response curves for alvimopan, and a dose of 12 mg does not give an increased GI recovery, although data were slightly more consistent through the trials (Table 1). This phenomenon is common for new drugs, particularly biologic agents, where effects on a receptor are not directly related to the concentration of the agent. These discrepancies may also be attributed to differing patient populations. However, a pooled analysis has shown that a 12 mg dose provided more consistent benefits across both sexes and all ages[54].

Alvimopan has had fewer side effects than placebo. Treatment adverse events reported in the published RCTs were most commonly nausea and vomiting, but these were less common in the alvimopan treated groups[60]. All studies on alvimopan have been conducted for open abdominal surgery.

Alvimopan will increase pharmacy expenditures; the cost of acquiring gum is substantially lower but more research is needed upon the effect of gum. Whether there is an additive effect upon GI recovery of pharmacological treatment (i.e. alvimopan and/or chewing gum) in combination with laparoscopic surgery and ERP protocols is unresolved.

LAPAROSCOPIC SURGERY AND POI

The greatest advance in limiting POI to date has probably resulted from the expanded use of laparoscopic surgery and the advantage of limiting tissue trauma. Recent studies suggest that laparoscopic surgery causes a lesser degree of mast cell activation and inflammation, and thus prolonged POI[61] (Figure 2). Laparoscopic surgery has many potential advantages over conventional open surgery, including smaller incisions, earlier GI recovery, shorter hospital stay and less pain[62].

Studies of large national databases suggest a higher rate for all commonly identified complications for open compared to laparoscopic colectomy. In a recent published study by Senagore *et al*[63], 4419 cases of open laparotomy were compared to 2728 cases of laparoscopy. All perioperative complications were more frequent in the open laparotomy group. Other database studies, identifying more than 30 000 cases of colectomies, shows superior results of laparoscopic colectomies compared to open cases[64]. Similarly, most other trials have demonstrated significant reductions in time to recovery of GI function after laparoscopic colectomy compared with open techniques, which translate into decreased hospital LOS. In a recent published study[28], mean GI recovery and LOS after laparoscopic colectomy were accelerated compared with those for patients in open laparotomy bowel resection.

Table 2 Effect of interventions to improve gastrointestinal recovery and reduce LOS

Trials	Type of study	Intervention	Improvement passage flatus	Improvement first bowel movement	Decrease in LOS
Noble *et al*[52]	Meta analysis	Chewing gum	14 h	23 h	1.1 d
Delaney *et al*[54]	Pooled analysis	Alvimopan	12 h[1] (6 mg) 15 h[1] (12 mg)	15 h[2] (6 mg) 18 h[2] (12 mg)	18.4 h[3]
Delaney *et al*[28]	Observational multicenter study	Laparoscopic surgery	NA	0.7 d[2]	1.7 d[3]
Walter *et al*[69]	Meta analysis	ERP	NA	NA	3.64 d

[1]Defined as GI[3]; [2]Defined as GI[2]; [3]Discharge order written. Standard elective open colorectal resection is usually associated with LOS of 8-12 d. Pharmacological treatment, laparoscopic surgery and ERP have substantially decreased LOS after surgery. LOS: Length of stay; ERP: Enhanced recovery pathways; NA: Not available.

The primary end points were time to upper and lower GI recovery (GI[2]: toleration of solid food and bowel movement) and postoperative LOS.

Overall POI-related morbidity (postoperative nasogastric tube insertion or investigator-assessed POI resulting in prolonged hospital stay or readmission) was similar between the open bowel resection and laparoscopic colectomy populations, suggesting that POI continues to cause significant morbidity regardless of the surgical approach[28].

Overall, there is substantial evidence that laparoscopic surgery helps accelerate GI recovery after surgery. Although the results in a study by Basse *et al*[65] did not show a benefit with laparoscopy, in our opinion the burden of evidence supports laparoscopic surgery as the standard approach when appropriate.

ERP AND POI

ERP have become part of the standardized postoperative recovery pathway at most hospitals. These protocols include many different elements and interventions, up to 20 elements in a recent published consensus review[66]. The main ERP elements are: avoidance of bowel preparation, preoperative fasting and carbohydrate loading, opioid sparing analgesia and mid thoracic epidural, antibiotic prophylaxis, laparoscopic surgery, small surgical incisions, no nasogastric tubes, normothermia, operative and postoperative fluid restrictions, no abdominal drains, suprapubic urinary drainage, oral diet at will, and early mobilization.

The benefits of fast track protocols (improved recovery, shortening of hospital stay, and earlier recovery of GI function) have been established in several RCTs[67]. Two systematic reviews of controlled and RCT supports the use of fast-track colorectal surgery[68,69]. ERP appear to be safe and shorten hospital stay 1-4 d after elective open colorectal surgery.

During the recent years, old dogmas have been challenged, such as keeping patients fasting after surgery. Allowing patients to eat normal food at will from the first day after major GI surgery does not increase morbidity, including the frequency of POI, when compared with traditional care with nil-by-mouth and enteral feeding[70]. LOS was reduced by approximately 3 d in the group allowed normal food at will. Similarly the use of a nasogastric tube after surgery has been debated. In fact routine nasogastric decompression does not accomplish any of its intended goals (including increased GI recovery) and so should be abandoned in favor of selective use of the nasogastric tube[71].

The best postoperative analgesic regime after surgery has been addressed in several trials, and is still debated. A recent published study advocates the use of thoracic epidural analgesia[72], however this conclusion is contested in a systematic review by Levy *et al*[73]. According to this review there is a paucity of data assessing the benefits of postoperative analgesic regimes following laparoscopic colorectal surgery and none of the protocols were shown to be clearly superior. Low *et al*[74] outlines the concerns of hypotension (increased risk of cardiovascular complications) and splanchnic hypoperfusion (increased risk of anastomotic leak) that are observed in connection with epidural analgesia. The best postoperative analgesic regime is therefore still unresolved; however epidural analgesic regime is included in most published ERP protocols, including a recent published consensus review[66]. Interestingly, while several randomized studies have suggested earlier recovery of GI function with epidurals, this has only been shown for opioid free epidurals, and has never translated into shorter hospital stay.

Over the last decade it has been increasingly realized that the pathophysiology of POI is multifactorial. Thus ideally ERP, and the care provided to colectomy patients should beneficially influence all four pathophysiologic pathways in the pathogenesis of POI (neurogenic, pharmacologic, hormonal, inflammatory, Figure 2)[75]. Although it is not yet clear which pathway is ideal, there is good consensus that fast track, ERP should be used at all modern hospitals, and included in all postoperative care.

FUTURE PERSPECTIVES

Standard elective colorectal resection is usually associated with a complication rate of 20%-30% and a postoperative stay of 8-12 d[67]. The introduction of pharmacological treatment, fast track protocols and laparoscopic surgery has changed this perspective (Table 2).

The introduction of laparoscopy in colorectal surgery improves early postoperative outcome, and randomized

trials have shown promising short term benefits with reduction of LOS by 3-4 d.

Despite rigorous research and several RCT trials during the past decade, the use of alvimopan is still debated, and for unclear reasons its incorporation into practice has been less than might be expected[76,77]. Several important questions remains unresolved, particularly the effect of alvimopan after laparoscopic surgery. After laparoscopic surgery, patients have less pain, lower opioid requirements and a shorter recovery time. It is unclear if alvimopan will have an additive effect upon the duration of POI and consequently LOS for this patient group.

Similarly, all of the alvimopan RCT studies were performed upon patients using a simple and standardized ERP protocol in the treatment and placebo groups. While ERP protocols may include up to 20 different elements, it is unclear which of these elements have the greatest impact upon POI and LOS. Similarly it is unclear what additive effect alvimopan may have upon POI and LOS for patients using ERP protocols. Recent studies[28,78,79] have however included fast track protocols in combination with laparoscopic surgery with promising results upon POI. Future POI research should address fast track protocols, laparoscopic surgery and pharmacological treatment, and the optimal combination of these interventions.

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EDITORIAL

Management of gallstones and gallbladder disease in patients undergoing gastric bypass

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Abstract

The appropriate management of gallstones and gallbladder disease in patients undergoing gastric bypass remains unknown. Several therapeutic modalities are used and include performing cholecystectomy on all patients at the time of gastric bypass, performing concomitant cholecystectomy only when patients have gallstones and performing cholecystectomy only in the presence of both symptoms and gallstones. Some groups administer ursodeoxycholic acid for gallstone prevention in the postoperative period. All treatment modalities are analyzed and their results and rationality are discussed.

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Key words: Gallbladder; Gastric bypass; Morbid obesity; Gallstones; Cholecystectomy

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INTRODUCTION

Rapid weight loss after bariatric surgery is one of many known risk factors for gallstone development, along with age, female gender, parity, race, obesity, genetics, very-low-calorie diets, short bowel syndrome, gallbladder motor dysfunction, diabetes, drugs and gastrointestinal surgery, among many others[1-25].

Traditionally cholecystectomy was indicated only in the presence of both gallstones and symptoms, but recently some have advocated elective cholecystectomy in selected cases in the absence of symptoms and even in the absence of gallstones[26-29].

Sustained weight loss after gastric bypass is achieved by a combination of gastric restriction and a variable degree of malabsorption[30-32] and has therefore a greater risk for gallstone development than purely restrictive procedures like adjustable gastric banding[33-35]. The appropriate management of gallstones and gallbladder disease in these patients is still under debate and several therapeutic modalities are used[36], including simultaneous cholecystectomy to all patients at the time of gastric bypass, regardless of the presence or absence of gallstones and/or symptoms (prophylactic approach)[37], simultaneous cholecystectomy only to patients with gallstones (elective or selective approach)[38] and expectant management with or without the prophylactic administration of ursodeoxycholic acid until symptoms develop (conventional approach)[39].

The objective of the paper is to discuss the rationale and the results obtained with these therapeutic modalities.

PROPHYLACTIC APPROACH

This consists of performing simultaneous cholecystectomy on all patients at the time of gastric bypass, regardless of the presence or absence of gallstones and/or symptoms.

The rationale behind this approach is based on the elevated incidence of gallstone development after gastric bypass compared to the normal population and the low sensitivity and specificity of ultrasonography for detecting gallstones in patients with morbid obesity[37,40-42]. A minimal morbidity rate with the addition of cholecystectomy is required.

In the series of Fobi *et al*[37] abnormal findings in the gallbladder were found in 75% of surgical specimens despite a negative preoperative ultrasound. Most of these patients had gallstones but other findings include cholesterolosis and cholecystitis. The addition of cholecystectomy to open gastric bypass added only an average of 15 min and the authors report no specific morbidity related to it.

Nougou *et al*[43] found some pathology in nearly 82% of specimens after simultaneous cholecystectomy with laparoscopic gastric bypass. 8.3% of patients did not undergo simultaneous cholecystectomy because it was judged to be dangerous. In the remaining patients, cholecystectomy added only 19 min on average to the procedure, with no extra ports addition. The authors do not report specific morbidity related to cholecystectomy.

Guadalajara *et al*[41] performed simultaneous cholecystectomy on 89 patients undergoing open gastric bypass and found a postoperative incidence of gallstones of 24% while the preoperative ultrasound incidence was only 16%.

Liem *et al*[44] performed simultaneous cholecystectomy on all patients undergoing open gastric bypass and found an incidence of gallbladder pathology of 80%.

A summary of these results is presented in Table 1.

ELECTIVE/SELECTIVE APPROACH

This consists of performing simultaneous cholecystectomy only on patients with asymptomatic gallstones diagnosed pre or intraoperatively.

The rationale behind this approach is based on an assumed higher incidence of symptomatic gallbladder disease in patients with gallstones in comparison to those without them. Some groups administer prophylactic ursodeoxycholic acid to patients without gallstones and therefore not submitted to concomitant cholecystectomy. A low morbidity rate is also required to support this approach.

Hamad *et al*[38] performed simultaneous cholecystectomy on 16.9% of patients at the time of gastric bypass. Operative times were significantly longer for patients undergoing simultaneous cholecystectomy and total hospital stay was almost doubled in comparison to gastric bypass without concomitant cholecystectomy. A significantly higher major morbidity rate was observed for patients undergoing simultaneous cholecystectomy but no specific morbidity was directly related to it. The most common pathological finding in the specimens was cholecystitis (99% of the cases). All patients without simultaneous cholecystectomy received 300 mg of ursodeoxycholic acid twice a day orally for a 6 mo period. During follow up 2.3% of these patients developed symptomatic gallstone disease and required cholecystectomy after an average of 12.4 mo.

Villegas *et al*[45] performed simultaneous cholecystectomy on 14% of patients after intraoperative diagnosis of gallstones or sludge with the aid of laparoscopic ultrasound. The global need for a subsequent cholecystectomy was 7%. Patients completing prophylactic ursodeoxycholic acid treatment had a significantly lower need of subsequent cholecystectomy.

In the series of open gastric bypass of Caruana *et al*[46] the diagnosis of gallstones was made by intraoperative palpation of the gallbladder. The authors did not report significant morbidity related to the addition of cholecystectomy. A subgroup of 125 patients that did not undergo simultaneous cholecystectomy was followed for at least 16 mo, requiring 8% of them to have a subsequent cholecystectomy for symptomatic gallstone disease.

Ahmed *et al*[47] retrospectively analyzed a series of 400 consecutive patients and found only significant differences in terms of operative times, which were 29 min longer for patients undergoing simultaneous elective cholecystectomy. No information about the incidence of symptomatic gallstone disease in the population without simultaneous elective cholecystectomy is given.

The group of the Universidad Católica de Chile reports a rate of simultaneous elective cholecystectomy of 10.9%[48]. The only significant differences were found for operative times, which were higher for the population undergoing simultaneous elective cholecystectomy. No information regarding the incidence of symptomatic gallstones in the population without simultaneous elective cholecystectomy was given.

Taylor *et al*[49] performed simultaneous cholecystectomy on 15% of patients. They reported the lowest need for subsequent cholecystectomy without the administration of prophylactic ursodeoxycholic acid, with only 3% of patients requiring it.

Tucker *et al*[50] performed simultaneous cholecystectomy on 7.2% of patients. A subgroup of patients was not submitted to this approach although they had gallstones present at the time of gastric bypass. The need for subsequent cholecystectomy in these patients was 17.6% whereas for patients without gallstones at the time of gastric bypass it was 6%.

A summary of these results is presented in Table 1.

CONVENTIONAL APPROACH

This consists of performing cholecystectomy only in the presence of both gallstones and symptoms, following the present guidelines for gallstone disease management[51,52]. The rationale behind this approach is to

Table 1 Results for cholecystectomy

Author	Yr	Indication for cholecystectomy	Increased morbidity	Ursodesoxicolic acid administration	Need for subsequent cholecystectomy
Fobi *et al*[37]	2002	Prophylactic open	No	NA	NA
Nougou *et al*[43]	2008	Prophylactic laparoscopic	No	NA	NA
Guadalajara *et al*[41]	2006	Prophylactic open	No	NA	NA
Liem *et al*[44]	2004	Prophylactic open	No	NA	NA
Hamad *et al*[38]	2003	Selective laparoscopic	Yes	Yes	2.30
Villegas *et al*[45]	2004	Selective laparoscopic	NR	Yes	7.00
Caruana *et al*[46]	2005	Selective open	No	No	8.00
Ahmed *et al*[47]	2007	Selective laparoscopic	No	No	NR
Escalona *et al*[48]	2008	Selective laparoscopic	No	No	NR
Taylor *et al*[49]	2006	Selective open	NR	No	3.00
Tucker *et al*[50]	2008	Selective laparoscopic	No	NR	11.80
Swartz *et al*[53]	2005	Conventional	NA	Yes	14.70
Fuller *et al*[54]	2007	Conventional	NA	Yes	7.69
Ellner *et al*[55]	2007	Conventional	NA	No	9.00
Portenier *et al*[56]	2007	Conventional	NA	No	8.10
Papasavas *et al*[39]	2006	Conventional	NA	No	7.83
Patel *et al*[57]	2006	Conventional	NA	No	6.00
Patel *et al*[58]	2009	Conventional	NA	No	4.90
Cosme Argerich Hospital	2010	Conventional	NA	No	9.84

NA: Not applicable; NR: Not reported.

indicate cholecystectomy only for the patients requiring it and since most of the subsequent cholecystectomies are performed when a significant weight loss is achieved, the operation is done in a leaner and healthier patient.

Swartz *et al*[53] found an incidence of subsequent cholecystectomy of 14.7%, with a significant lower incidence for patients completing prophylactic ursodeoxycholic acid treatment. In Fuller and coworker's experience, the need for subsequent cholecystectomy in patients completing prophylactic ursodeoxycholic acid treatment was 7.69%[54].

Ellner *et al*[55] and Portenier *et al*[56] did not administer prophylactic ursodeoxycholic acid treatment and found an incidence of subsequent cholecystectomy of 9% and 8.1%, respectively.

Papasavas *et al*[39] did an interesting study in which they retrospectively reviewed the records of 644 patients undergoing gastric bypass without simultaneous cholecystectomy. All of them received postoperative prophylactic ursodeoxycholic acid treatment. The need for a subsequent cholecystectomy in patients with gallstones present at the time of gastric bypass was 8.3%, which is similar to the incidence observed for patients without gallstones (6.9%) and to the incidence for patients without preoperative ultrasound screening (8.3%). After obtaining these results, this group no longer screens for gallstones preoperatively. Similarly, Patel *et al*[57] at the UCLA School of Medicine found an incidence of subsequent cholecystectomy without preoperative screening and without postoperative ursodeoxycholic acid administration of 6%. A subsequent analysis by the same group with a longer follow up showed an even lower rate of subsequent cholecystectomy of 4.9%[58].

In our experience at Cosme Argerich Hospital from Buenos Aires, the global need for cholecystectomy after gastric bypass, without prophylactic ursodeoxycholic acid treatment, was 9.84% (unpublished data). For patients with gallstones present at the time of surgery a subsequent cholecystectomy was needed in 5% and for patients without gallstones in 10.71% (*P*: not significant). Based on our own data, the natural history of patients with asymptomatic gallstones undergoing gastric bypass is very much like the natural history of asymptomatic gallstones in the general population[59-61].

A summary of these results is presented in Table 1.

URSODEOXYCHOLIC ACID TREATMENT

The preventive administration of ursodeoxycholic acid proved to be significantly better than placebo in preventing gallstone formation in a double blind, prospective and randomized study conducted by Sugerman *et al*[62]. A daily dose of 600 mg was associated with the lowest rate of gallstone formation and the lowest incidence of adverse events. Patients that developed gallstones showed a lower compliance rate. The effect of the 6 mo treatment seems to last for at least 1 year, since at that moment patients were reevaluated with ultrasonography and the incidence of gallstones was significantly less compared to the placebo arm. Unfortunately, there is no mention in the study regarding how many of those patients that developed gallstones were actually symptomatic and therefore required cholecystectomy, since the actual standard of care for non-obese patients indicates a cholecystectomy only when both symptoms and gallstones are present (SSAT NIH). A true benefit for ursodeoxycholic acid would be a lower rate of delayed cholecystectomy over the placebo group.

Wudel *et al*[63] compared, in a randomized double-blind fashion, a cohort of 60 patients without gallstones at the time of open gastric bypass and prescribed them

a 6-mo course of ursodeoxycholic acid, ibuprofen or placebo. 71% of the patients subsequently developed gallstones and no benefit of the two therapies investigated could be demonstrated because of an extremely low compliance rate of 28%.

A recently published meta-analysis by Uy *et al*[64] concluded that ursodeoxycholic acid administration prevents gallstone formation after bariatric surgery, but no meta-analysis of symptomatic gallstones could be done, since only one paper addressed this topic and unfortunately did not include patients undergoing gastric bypass[65].

CONCLUSION

Prophylactic and selective management can be safely performed and the only significant difference with patients not submitted to concomitant cholecystectomy is mostly observed in operative times that are higher in those who do undergo cholecystectomy. Data obtained from conventional management studies, with or without ursodeoxycholic acid administration, show that most of the patients remain asymptomatic even when they develop gallstones and therefore do not require a subsequent cholecystectomy, so that the risks of performing a concomitant cholecystectomy might be unwarranted. Treatment with ursodeoxycholic acid prevents gallstone formation after gastric bypass but most of the studies conducted show a low compliance rate and do not mention the true benefit of the treatment, which would be a lower cholecystectomy rate.

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TOPIC HIGHLIGHT

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Connection between inflammation and carcinogenesis in gastrointestinal tract: Focus on TGF-β signaling

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Abstract

Inflammation is a primary defense process against various extracellular stimuli, such as viruses, pathogens, foods, and environmental pollutants. When cells respond to stimuli for short periods of time, it results in acute or physiological inflammation. However, if the stimulation is sustained for longer time or a pathological state occurs, it is known as chronic or pathological inflammation. Several studies have shown that tumorigenesis in the gastrointestinal (GI) tract is closely associated with chronic inflammation, for which abnormal cellular alterations that accompany chronic inflammation such as oxidative stresses, gene mutations, epigenetic changes, and inflammatory cytokines, are shared with carcinogenic processes, which forms a critical cross-link between chronic inflammation and carcinogenesis. Transforming growth factor (TGF)-β is a multi-potent cytokine that plays an important role in regulation of cell growth, apoptosis and differentiation. Most importantly, TGF-β is a strong anti-inflammatory cytokine that regulates the development of effector cells. TGF-β has a suppressive effect on carcinogenesis under normal conditions by inhibiting abnormal cell growth, but on the other hand, many GI cancers originate from uncontrolled cell growth and differentiation by genetic loss of TGF-β signaling molecules or perturbation of TGF-β adaptors. Once a tumor has developed, TGF-β exerts a promoting effect on the tumor itself and stromal cells to enhance cell growth, alter the responsiveness of tumor cells to stimulate invasion and metastasis, and inhibited immune surveillance. Therefore, novel development of therapeutic agents to inhibit TGF-β-induced progression of tumor and to retain its growth inhibitory activities, in addition to anti-inflammatory actions, could be useful in oncology. In this review, we discuss the role of TGF-β in inflammation and carcinogenesis of the GI tract related to abnormal TGF-β signaling.

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Key words: Inflammation; Carcinogenesis; Transforming growth factor-β; Gastrointestinal tract

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TRANSFORMING GROWTH FACTOR-β IN HOMEOSTASIS AND INFLAMMATION OF GASTROINTESTINAL TRACT

The gastrointestinal (GI) tract is composed of the esophagus, stomach, small intestine and large intestine; it is the longest and largest surface among human organs, and is the first contact place for various pathogens and food antigens. Therefore, the GI tract should have many lymphoid organs to control the untoward or harmful immune reactions. The immune system of the GI tract can induce a series of host defensive reactions such as recruitment of immune cells, activation of anti-inflammatory enzymes, and secretion of anti-inflammatory cytokines[1]. To prevent pathological inflammation against various antigens exposed to the GI tract, the human body has developed various regulatory mechanisms: the development of regulatory T (Treg) cells, the maintenance of the gut barrier, and immune reactions. Since the inflammatory process in the GI tract is started by food antigens, extracellular pollutants and pathogenic infections, disruption of the intestinal barrier results in intestinal inflammation by inducing the pro-inflammatory reactions of immune cells[2].

Therefore, in order to maintain homeostasis of the GI tract, tolerance is a prerequisite to maintain the balance between pro-inflammation and anti-inflammation. Although many types of regulatory immune cells perform their suppressive functions in the GI tract to acquire tolerance, this activity is mediated by a relatively small number of materials including interleukin (IL)-10 and transforming growth factor (TGF)-β, which are well known regulators of immune reactions in the GI tract[3,4].

TGF-β is a multifunctional cytokine with critical roles in many cellular pathways including cell growth, apoptosis, differentiation and immune reactions[5,6]. TGF-β is secreted as a latent inactive protein and needs to be activated *via* conformational change or protein cleavage by protease or thrombospondins. Since TGF-β1 knockout mice show a dramatic phenotype and develop severe autoimmunity that leads to death within 2 wk after birth[7,8], and T-cell specific disruption of TGF-β signaling results in serious inflammatory changes through constitutively activated T cells in the gut and lung, as a similar phenotype to whole TGF-β knockout mice[9], TGF-β is thought to be a strong anti-inflammatory cytokine. In the regulation of intestinal inflammation, TGF-β inhibits T-cell proliferation as well as blocking differentiation of $CD4^+$ and $CD8^+$ naïve T cells into helper T cells by inhibiting expression of the transcription factors such as T-bet, STAT4 and GATA-3[10]. Furthermore, TGF-β suppresses immune reactions through the induction of Treg cells. In fact, TGF-β has been shown to be essential for the induction and maintenance of peripheral $CD4^+CD25^+$ Treg cells by activation of Foxp3 expression[11].

However, TGF-β also has pro-inflammatory effects through the differentiation of Th17 cells by induction of retinoic-acid-receptor-related orphan nuclear receptor γt, a Th17-specific transcription factor[12]. Conclusively, cross-talk with surrounding tissues may be important for activity of TGF-β in GI tract inflammation. TGF-β has a critical role in regulation of inflammatory processes, therefore, it should be tightly regulated by various mechanisms. Dysregulated or attenuated TGF-β signaling has been suspected in the pathogenesis of various inflammation-related diseases including chronic inflammatory disorders and cancer. T-cell specific deficiency of furin, which activates latent TGF-β, leads to spontaneous autoimmune disease such as colitis and intestinal inflammation in murine models[13]. Similarily, overexpression of mutant TGF-β, which has a defect in binding activity with integrins, shows similar phenotypes to TGF-β null mice, such as vascular defects, multi-organ inflammation, and lack of Langerhans cells[14].

Disruption of Smad3 in mice also shows defects in mucosal immunity and results in early death after birth. Smad3 mutant mice exhibit large amounts of infiltration of T cells and bacterial abscess formation in the GI tract[15]. Activation of Smad3 in patients with inflammatory bowel disease (IBD) is diminished compared to unaffected persons. Isolated cells from IBD patients do not respond to treatment with TGF-β, and do not activate phosphorylation of Smad3, even in the presence of high concentrations of TGF-β[16,17]. In addition, interaction between Smad3 and Smad4 is markedly decreased in lamina propria mononuclear cells of IBD patients. All of these pathogenic phenotypes seem likely to originate from overexpression of Smad7, which is inhibitory for TGF-β signaling. The anti-inflammatory activity of TGF-β comes from inhibition of nuclear factor (NF)-κB activation, but it is lost in the intestine of IBD patients due to high levels of Smad7[18]. An *in vitro* mouse model of colitis treated with trinitrobenzene sulfonic acid or oxazolone shows similar results to those in humans[19]. When anti-sense oligonucleotide against Smad7 is given to mice to reduce the level of Smad7, TGF-β-induced phosphorylation of Smad3 is markedly increased. Conversely, the inflammatory phenotypes of the colon, such as weight loss and microscopic changes, are recovered to normal states after treatment with Smad7 antisense nucleotides. These data indicate that precise regulation of TGF-β signaling, including activation of Smad3 and level of Smad7, is important for homeostasis during inflammation of the GI tract. In contrast to these data, Smad7 also has anti-inflammatory activity against tumor necrosis factor (TNF) signaling and mediates the suppressive activity of TGF-β in primary macrophages and mouse skin[20]. This discrepancy might originate from

differences in cell lines, mouse strains, and experimental systems.

TGF-β LIGANDS AND THEIR SIGNALS

TGF-β sends signals through the heterodimeric complex formation of type I and type Ⅱ serine/threonine kinase receptors. When the ligands interact with receptor Ⅱ, type I receptor is activated by phosphorylation of serine residues. Activated receptor complexes recruit the adaptor proteins, receptor-activated Smad proteins (R-Smad). For example, Smad2 and Smad3 are R-Smad proteins for TGF-β and Smad1, Smad5 and Smad8 are R-Smad proteins for bone morphogenic protein signaling. R-Smad proteins have two conserved domains that are separated by a middle linker domain. The MH1 domain of the N-terminal region functions as a DNA-binding domain and the C-terminal of the MH2 region regulates the nuclear localization and transcriptional activity of R-Smad proteins. Receptor-mediated phosphorylation of C-terminal serine residues induces activation and nuclear translocation of R-Smad proteins. Activated R-Smad proteins form a complex with common Smad (co-Smad) protein, Smad4. The interaction between R-Smad and co-Smad proteins is mediated through the MH2 domain and induces the translocation into the nucleus of Smad complexes. In the nucleus, Smad complexes interact with Smad binding elements (SBEs) of target genes and regulate the transcriptional activity. TGF-β upregulates or downregulates the expression of target genes, which depend on Smad-interacting partners, coactivators or corepressors[6].

CROSS-LINKING BETWEEN INFLAMMATION AND GI CARCINOGENESIS

Relationship between chronic inflammation and carcinogenesis was originally suggested by Virchow, who first hypothesized in 1893 that the lymphoreticular infiltrate may be the origin of tumor at sites of chronic inflammation. Over 100 years later, his hypothesis was cited as evidenced that chronic inflammatory conditions definitely promote progression of malignant tumors. Inflammatory cells and cytokines act as a tumor promoter that affects cell survival, proliferation, invasion, angiogenesis and chemo-resistance[21-23]. Almost 15% of cancers are reported to occur through chronic inflammation-related processes (Table 1). We have established animal models of GI tumors, all of which were based on the concepts that inflammation leads to tumorigenesis, Barrett's esophagus originates through repeated reflux-induced inflammation in the esophagus, colitic tumors arise from repeated bouts of colitis, and gastric cancer is associated with *Helicobacter pylori* (*H. pylori*) infection.

Reflux esophagitis and esophageal cancer

More than 500 000 patients are diagnosed with esophageal cancer annually, which has the second highest mortality after pancreatic cancer among GI cancers worldwide. The most typical type of esophageal cancer is, in histological terms, squamous epithelial carcinoma, and Asians have a greater prevalence of this histological type of carcinoma than adenocarcinoma. On the other hand, Caucasians have just as many cases of adenocarcinoma as squamous carcinoma[24,25]. Many reasons to explain such differences can be put forward, including lifestyle and genetic factors, but in particular, Barrett's esophagus results in a great discrepancy in incidence between Western and Asian populations. Since the numbers of reflux esophagitis patients are rapidly increasing in Korea, as lifestyle as well as living environment have changed to more western styles, in the near future, it is expected that esophageal adenocarcinoma will be more prevalent among Koreans. Esophageal adenocarcinoma is closely related to Barrett's esophagus, therefore, carcinogenesis has the order of chronic reflux esophagitis followed by Barrett's esophagus and esophageal adenocarcinoma. Reflux esophagitis leads to chronic esophagitis and an increase in Barrett's esophagus, which directly creates a precancerous lesion, for which we have demonstrated that chronic exposure to gastroduodenal contents through duodenoesophageal anastomosis leads to metaplastic changes in the squamous epithelium (Figure 1). Molecular factors related to reflux esophagitis are as follows: increase in cyclooxygenase-2 (COX-2), IL-8, IL-1β, IL-10 and TNF-α; loss of TGF-β signaling; and activation of NF-κB. All of these contribute to development of Barrett's esophagus. This mediates chronic inflammation that increases expression of CDX-like homeobox family genes and the caudal gene family, which results in intestinal metaplasia of squamous epithelial cells. Intestinal metaplasia is the direct cause of esophageal adenocarcinoma and because it shares similar molecular mediators with chronic esophagitis, it is thought to be involved in carcinogenesis[26,27]. Barrett's esophagus is thought to be a precancerous lesion because of the appearance of the following changes: augmentation of the cell cycle and proliferation, increased angiogenesis and aneuploidy, decreased antiproliferative signaling and apoptosis, accompanied by the histological changes involved in the progression from chronic esophagus to Barrett's esophagus.

Table 1 GI tumorigenesis associated with chronic inflammation

	Tumor	Causes
Infection-related		
Chronic gastritis	Gastric cancer	*H. pylori*
Chronic gastritis	Gastric cancer	Cytomegalovirus
Prostate inflammatory atrophy	Prostate cancer	Pathogens
Chronic hepatitis	Hepatocellular cancer	HBV and HCV
Infection-unrelated		
Esophagitis	Esophageal cancer	Gastric acid, alcohol, tobacco
Pancreatitis	Pancreatic cancer	Alcohol, tobacco
IBD	Colorectal cancer	Crohn's disease, UC
Cholecystitis	Gall bladder cancer	Gall bladder stone

GI: Gastrointestinal; *H. pylori*: *Helicobacter pylori*; HBV: Hepatitis B virus; HCV: Hepatitis C virus; UC: Ulcerative colitis; IBD: Inflammatory bowel disease.

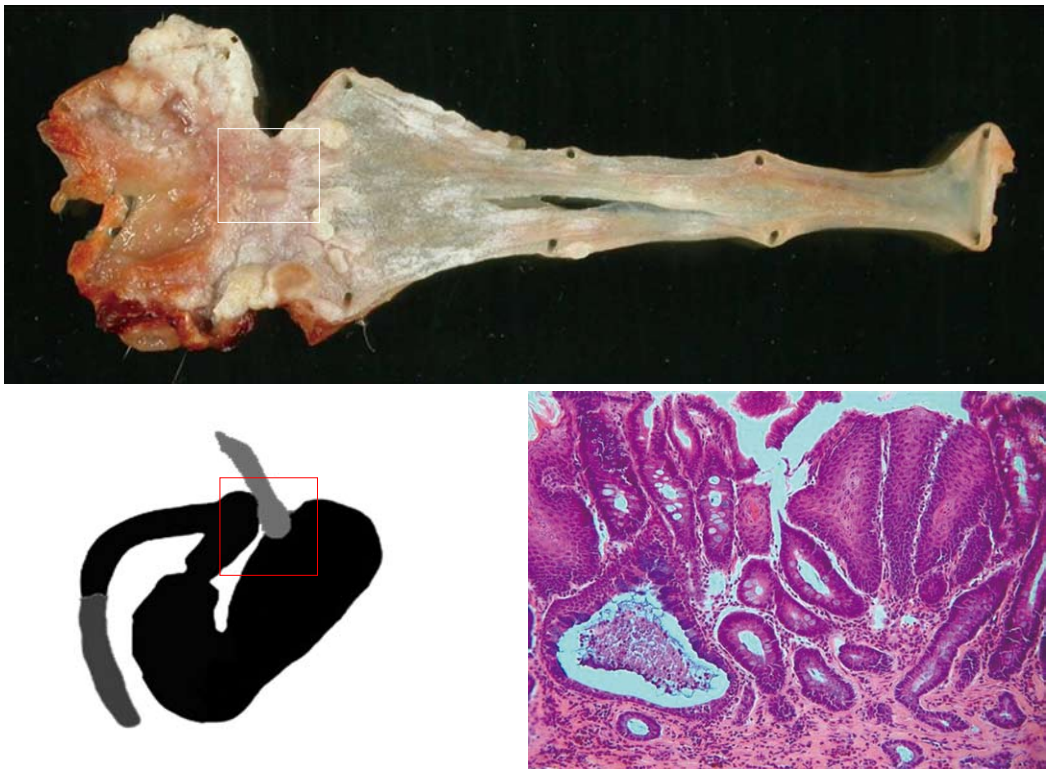


Figure 1 Connection between inflammation and carcinogenesis as demonstrated in a rat model of Barrett's esophagus. With the application of surgical bypass, esophagoduodenostomy (red boxed), overt discolored lesions (white boxed) were noted at the esophagogastric junction, which showed the existence of intestinal metaplasia against a background of squamous epithelium. These animal models provided evidence of the cross-linking between inflammation and premalignant lesions.

Chronic pancreatitis and pancreatic cancer

Pancreatic cancer has the poorest prognosis among GI cancers because patients are diagnosed at a fully progressed stage, and the tumor cells are very aggressive, so that metastasis is very frequent and they do not respond to anti-cancer therapy. Chronic pancreatitis patients have a 3.8-18.5 times higher chance of developing pancreatic cancer[28]. However, severe chronic pancreatitis cannot be discriminated from pancreatic cancer, especially in cases with a history of smoking and alcohol consumption, and a family history. Continuation of chronic pancreatitis for long periods can create a cancer-like molecular environment, such as activation of COX-2, NF-κB and inducible nitric oxide synthetase, production of cytokines such as IL-1, IL-6, IL-8 and TNF-α, and free radical oxygen formation[29,30]. TGF-β plays such an important role in regulating onset of chronic pancreatitis, and we have shown that lack of TGF-β in pancreatic acinar cells leads to increased sensitivity to acute pancreatitis, as well as a favorable environment for chronic pancreatitis.

Chronic atrophic gastritis and gastric cancer

Although there has been a tendency towards a decrease in gastric cancer for the past 80 years, it is still the second highest cause of death following lung cancer. In Korea, about 25-30 people out of every 100 000 develop gastric cancer regardless of their sex, and its mortality rate is ranked as the highest. However, the discovery of a causative pathogen of gastric cancer, *H. pylori*, has raised hopes for preventing gastric cancer through eradication or regulation of the bacterium. The International Agency for Research on Cancer in 1994 defined *H. pylori* as an imminent carcinogenic pathogen, based on epidemiological research and animal studies. This definition is based on epidemiological evidence, and several animal experiments have shown that infection with *H. pylori* causes infiltration of inflammatory cells, oxidative damage and gene mutations. In resected specimens from gastric cancer, the intestinal metaplastic lesions are easily identified around the cancer lesions, with which *H. pylori* infection is closely associated. Although *H. pylori* is a class I carcinogen, gastric cancer is not prevented by *H. pylori* eradication in all patients. This can be explained by the relationship between gastritis, metaplasia and gastric cancer, whereby prevention of *H. pylori*-associated carcinogenesis only benefits those in whom the malignant process has not begun[31]. That is, even though *H. pylori* is completely eradicated, gastric inflammation remains, thus it seems that amelioration of gastric inflammation itself seems to be far more essential in achieving cancer prevention than eradication of the pathogen. This emphasizes that chronic gastritis has a greater chance of causing onset of gastric cancer than the presence of *H. pylori* itself. Furthermore, genetic factors, toxicity of the pathogen, and environmental factors are intertwined in the development of *H. pylori*-induced gastric cancer from inflamma-

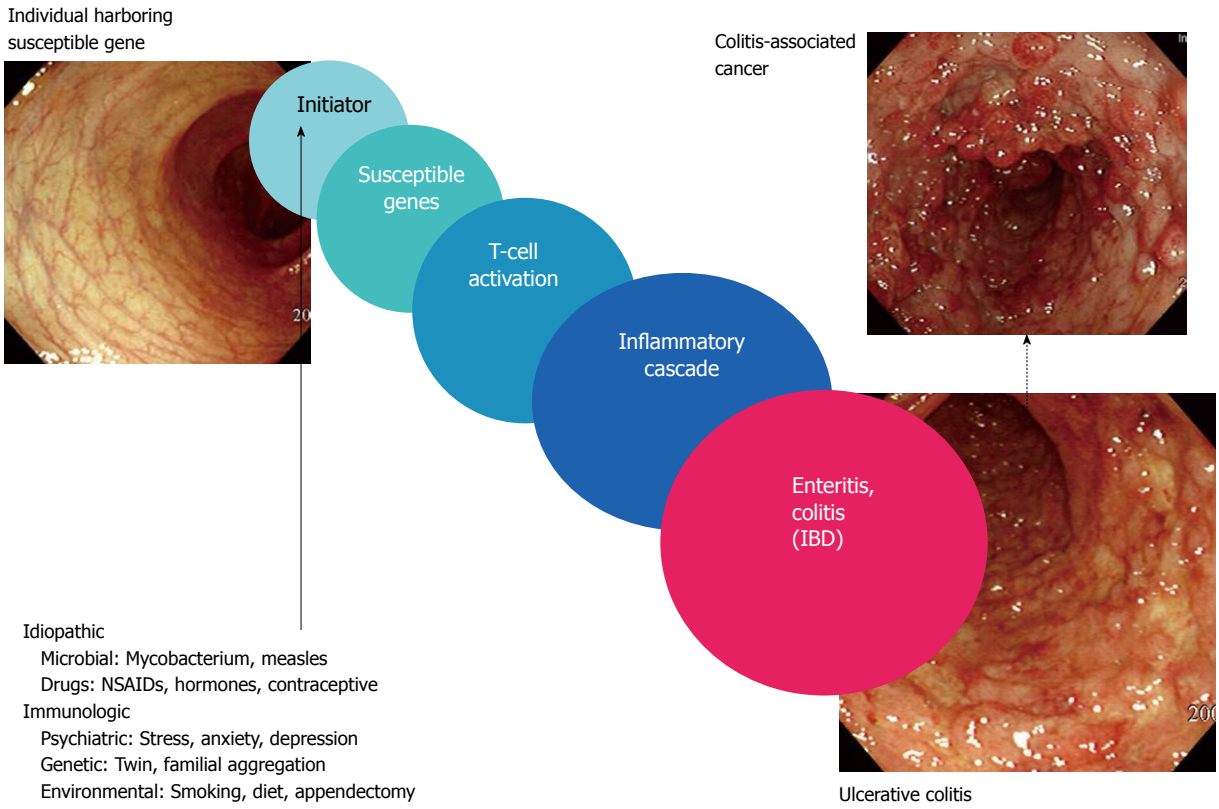


Figure 2 Inflammatory bowel disease (IBD) and colitis-associated cancer. The cause of IBD is idiopathic because several etiologies have been suggested including microorganisms, drugs, psychiatric illness, genetic abnormality, and environmental factors. However, when individuals that harbor IBD susceptibility genes are exposed to the above-mentioned etiological factors, they experience T cell activation, after which perpetual inflammatory cascades lead to enteritis or colitis. Repeated bouts of chronic colitis alone provoke colitis-associated cancer, which emphasizes the cross-linking between inflammation and carcinogenesis. NSAIDs: Nonsteroidal antiinflammatory drugs.

tion, thus alleviation or treatment of inflammation could form the basis of prevention of gastric cancer[32,33]. The contribution of TGF-β to *H. pylori*-associated gastric carcinogenesis has been investigated using pS2-dnR Ⅱ mice, which are defective in TGF-β signaling due to expression of the dominant negative R Ⅱ receptor (TGF-β-dnR Ⅱ) in the stomach, using gastric specific promoter pS2 (gastric trefoil peptide). pS2-dnR Ⅱ mice developed gastric adenocarcinoma with *H. pylori* alone compared to their wild-type littermates, which suggests that the development of gastric cancer after *H. pylori* infection might be influenced by host genetic conditions[34].

IBD and colitis-associated cancer

The most common cause of colorectal cancer is sporadic. However, 10%-15% of these patients continually suffer from chronic IBD for 10 years, which later develops into colitis-associated cancer, namely, colitic cancer. Ulcerative colitis (UC) is a form of chronic IBD that usually has a clinical course of repeated exacerbation and remission, and less commonly presents with an unremitting, fulminating course. Another feature of the clinical course of UC is an increase risk for the development of colitic cancer (Figure 2). Although sporadic colorectal cancer and colitic cancer arise from dysplastic precursor lesions and share several molecular alterations, the nature of the dysplasia and the frequency and timing of several of the key molecular changes differ sufficiently to support our hypothesis that colitic cancer can be prevented by early intervention with strong anti-inflammatory agents or cancer surveillance with strict biomarkers. Etiologically, in the case of sporadic colorectal cancer, most cases are preceded by adenomatous polyps, namely adenoma, followed by the so-called "adenoma-carcinoma" sequence[35]. On the other hand, colitic cancer is contracted at a relatively young age and the crucial factor is the range, extent and period of inflammation, which implies the "inflammation-dysplasia-carcinoma" sequence. Considering the fact that the number of IBD patients is increasing, in the forthcoming 10 years, inflammation-related cancer is expected to increase, thus we should be focusing on cancer prevention through control of inflammation. We have studied colorectal cancer prevention based on an animal model of inflammation-related cancer, and are working on how the control of inflammation itself prevents colorectal cancer. We have performed another similar animal model with repeated bouts of colitic cancer and have shown that potent anti-inflammatory drugs inhibit cancer formation. This suggests that colitis is involved at the beginning as well as the progression of cancer, and control of inflammation is one of the methods for preventing inflammation-related colorectal cancer.

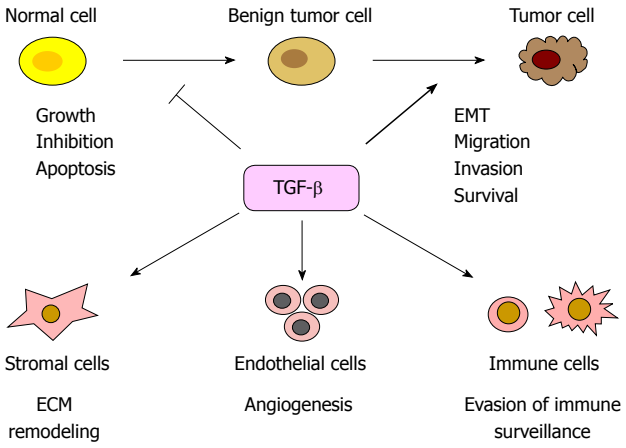


Figure 3 Role of transforming growth factor (TGF)-β in cancer progression. TGF-β is a secreted polypeptide that signals *via* receptor serine/threonine kinases and intracellular Smad effectors. TGF-β inhibits proliferation and induces apoptosis in various cell types. Accumulation of loss-of-function mutations in the TGF-β receptor or Smad genes classifies the pathway as a tumor suppressor in humans. In addition, various oncogenic pathways directly inactivate the TGF-β receptor-Smad pathway, thus favoring tumor growth. On the other hand, all human tumors overproduce TGF-β whose autocrine and paracrine actions promote tumor cell invasiveness and metastasis. Accordingly, TGF-β induces epithelial to mesenchymal transition (EMT).

TGF-β IN GI CARCINOGENESIS

TGF-β signaling is a crucial regulator of intestinal homeostasis and inflammation, therefore, dysregulation of this pathway could be associated with inflammation-related carcinogenesis of the GI tract (Figure 3). Altered signaling pathways can disturb the regulatory mechanism of TGF-β to block chronic inflammation followed by carcinogenesis. As a tumor suppressor gene, TGF-β can inhibit initiation of cell transformation, through growth inhibition and apoptosis in normal epithelium. During changes in the genetic and epigenetic context of transforming cells, the responsiveness of cells to TGF-β is decreased, but the expression and activation of TGF-β are markedly increased[36].

Mouse models have been used to investigate the role of TGF-β in GI inflammation and tumorigenesis. Heterozygotic TGF-β null mice express only 10%-30% of wild-type TGF-β1 protein level, and show increased cell turnover with chemical carcinogens, which results in enhanced tumorigenesis compared with wild-type mice[37]. Furthermore, TGF-β1/Rag2 double knockout mice can live for a long time and develop colon cancer after developing inflammatory lesions[38]. Mutations of TGF-β receptor Ⅱ (TGF-β-RⅡ) are frequently found in colon and gastric cancer with microsatellite instability[39]. Conditional transgenic mice using TGF-β-dnRⅡ with pS2/TFF1 promoter were created and infected with *H. pylori*, and they developed dysplasia as well as adenocarcinoma, which suggests that disruption of TGF-β signaling is related to gastric cancer formation[34]. The findings that TGF-β-dnRⅡ mice have higher levels of gastritis than their wild-type littermates, suggests that TGF-β1 plays an important role in prevention of gastric inflammation as well as cancer. An experiment for inducing IBD was performed on mice that overexpressed TGF-β-dnRⅡ in the small/large intestine using the promoter of ITF (intestinal trefoil factor)/TFF3. TGF-β-dnRⅡ mice showed much more severe colitis than their wild-type littermates when induced with the same concentration of dextran sulfate sodium[40].

Some pancreatic and biliary adenocarcinomas originate from mutation of TGF-β receptor Ⅰ (TGF-β-RⅠ) with very low frequency[41]. In a mouse model, mutation or deletion of bone morphogenetic protein receptor, which is another member of the TGF-β superfamily, also induces chronic inflammatory phenotypes and then precancerous lesions[42].

Deletion of Smad proteins also leads to loss of the regulatory role of immune suppression and induces malignant cell proliferation. Deficiency of Smad4 in mutant mice causes gastric polyps and progression to gastric tumor in old age[43]. In humans, deletion of Smad4 is found in juvenile polyposis syndrome and is a strong risk factor for GI cancer[44]. In addition, T-cell specific deletion of Smad4 results in spontaneous epithelial cancer of the oral cavity, stomach, colon, rectum and duodenum in mice[45]. Smad3 knockout mice also develop metastatic colorectal cancer through dysregulation of cell proliferation. The expression of Smad3 shows low to undetectable levels in 40% of human gastric cancer tissues[46]. Reintroduction of Smad3 into gastric cancer cells restored their responsiveness to TGF-β and blocks tumor formation in nude mice. These data support the suggestion that Smad-mediated TGF-β signaling regulates homeostasis and inflammation in the GI tract.

Mutations in fine tuning of regulatory circuits cause the loss of normal communication within target organs, and defects in regulating stromal and epithelial interactions. In addition to Smad proteins, cofactors of TGF-β signaling can also affect the biological roles of TGF-β and the progress of GI carcinogenesis. Based on mouse model studies, mutations of these cofactors, such as embryonic liver fodrin (ELF), promyelocytic leukemia tumor suppressor, Smad anchor for receptor activation, and filamin lead to malformation of intestinal structures and induce the formation of liver, gastric and colon cancers. In case of ELF, 40%-70% of heterozygotic null mice develop hepatocellular cancer within 15 mo through abnormal angiogenesis or cyclin D1 activation[47,48].

In contrast to its tumor suppressive activity, expression of TGF-β1 is markedly increased in colon, esophageal, gastric, hepatocellular and pancreatic cancer[49]. High levels of TGF-β expression are correlated with tumor progression, metastasis and angiogenesis, which results in poor prognostic outcome. In addition to its oncogenic nature related to TGF-β mutation, TGF-β, which is secreted from tumor cells, can control tumor progression by suppressing cytotoxic immune reactions, stimulating expression of cell survival factors, or regulating autonomous signaling of tumor cells, depending on cell type and context. At the late stage of tumor develop-

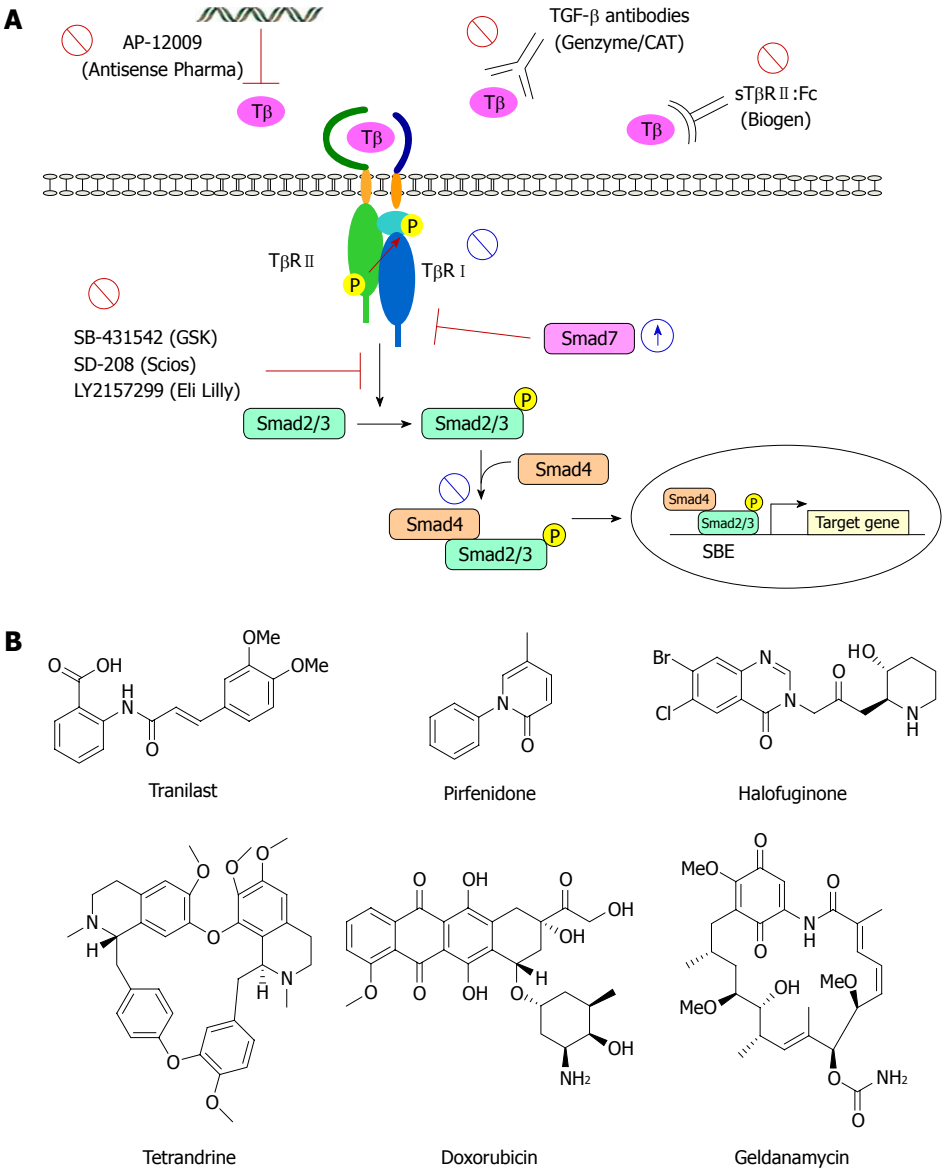


Figure 4 Therapeutic target of TGF-β signaling. A: TGF-β signaling and points of therapeutic intervention; B: Structures of multi-action TGF-β signal inhibitors. SBE: Smad binding element.

ment, invasive and metastatic potentials are important properties, and TGF-β signaling is a crucial pathway for tumor cell invasion and metastasis by inducing epithelial to mesenchymal transition (EMT)[50,51]. EMT progresses by decreasing the expression of E-cadherin to inhibit cell-cell adhesion and by increasing the expression of laminin-5, vimentin, integrins, and fibulin-5 that are involved in cell-extracellular matrix associations[52,53].

THERAPEUTIC TARGET OF TGF-β SIGNALING

Until now, the most successful target-aimed anti-cancer treatment has focused on the suppression of oncogenes that are abnormally activated in cancer cells. As examples, Gleevec® (imatinib), which is a *Bcr-abl* kinase inhibitors, and Herceptin (trastuzumab), which is a monoclonal antibody that interferes with the *Erb*B2/*Neu* receptor, are good examples of target-aimed molecular cancer treatments[54]. However, regarding TGF-β, the development of TGF-β inhibitors for the purpose of cancer treatment has shown no apparent progress so far in spite of TGF-β being known as a potent growth inhibitory factor against epithelial and blood cells. Instead, TGF-β signal transduction has been recognized as one of the representative targets of cancer treatment, aided by recent clinical results and cancer biology with regard to TGF-β. TGF-β ligands, as well as various downstream signal transducers can be a target in the therapeutic regulation of TGF-β signaling (Figure 4A). For instance, a potential target for the therapeutic intervention can be unveiled by inhibiting TGF-β synthesis, suppressing TGF-β activity at the cell surface, removing or neutralizing synthesized TGF-β, or regulating receptors and Smads involved in intracellular signaling pathways. By using EMT6 murine mammary tumor cell line that stably expresses antisense TGF-β transduced with a retroviral vector, it has been

demonstrated that the decreased production of TGF-β inhibited tumor growth[55]. In particular, the decreased secretion level of TGF-β by cancer cells induced tumor regression by increasing the activity of anti-tumor cytotoxic T cells. Currently, the inhibition of TGF-β production by using anti-sense oligonucleotides is one of the most potent methods in clinical cancer treatment. For example, AP-12009, which is synthesized by Antisense Pharma and is especially selective for TGF-β2, has prominent clinical effects on malignant neuroglial tumor patients[56,57]. It is now in phase Ⅱ clinical trials for the treatment of neuroglioma and pancreatic and skin cancer. The suppression of latent TGF-β activation can also be useful for regulating TGF-β activity. It has been reported that the furin protease inhibitor, decanoyl-Arg-Val-Lys-Arg-chloromethylketone (*dec-RVKR-cmk*), participates in TGF-β activity and inhibits the release of mature bioactive TGF-β molecules *in vitro*[58]. However, furin is not considered as a good selective inhibitor for TGF-β signaling because it is known to be involved in the activity of other growth factors, growth factor receptors, integrin, and matrix metalloproteinase.

Agents that promote TGF-β signaling

Restoration or augmentation of TGF-β signal transduction can be a good strategy for cancer prevention and treatment. For instance, chemical compounds such as retinoid and deltanoid (vitamin D), which are related to the expression or regulation of TGF-β signaling, have been used for chemoprevention of cancer[59,60]. Based on tumor suppressor activity of TGF-β, various attempts have been made to screen compounds that may activate TGF-β signaling. The first attempt, which was made by Seto's group, was to screen small molecular chemical compounds that have similar activities to TGF-β[61,62]. By searching for agents that increased the transcriptional activity of plasminogen activator inhibitor-1 (PAI-1), which is one of target genes of TGF-β, they finally isolated diheteropeptin and spiruchostatin A and B from microbial metabolites. These compounds turned out to increase the expression of PAI-1 and p21 and suppress the growth of lung epithelial cells in nanomolar concentrations. In addition, they had an inhibitory activity against histone deacetylase (HDAC). Lee *et al*[63] have isolated onnamide A and theopederin B from marine sponge, which promotes the transcriptional activity of TGF-β.

Onnamide A and theopederin B induce expression of PAI-1 gene and apoptosis through activation of mitogen-activated protein kinases (MAPKs) such as p38 and JNK, which suggests that they have strong anti-tumor activity. Moses's group[64] identified one TGF-β mimicking agent, A-161906, by performing high-throughput screening using mink lung epithelial cells stably transfected with PAI-1 promoter reporter. As an HDAC inhibitor, A-161906 demonstrated cellular effects similar to those of TGF-β. A-161906 not only inhibited cell proliferation, but also induced growth arrest at the G1-S checkpoint, and expression of the cyclin-dependent kinase inhibitor p21. In addition, based on the fact that Smad4 (DPC4) mutation, which is frequently found in pancreatic cancer, plays a crucial role in the loss of TGF-β-mediated growth inhibition, Sohn's group[65] performed high-throughput screening. Using a stably transfected reporter gene that has six copies of SBE (p6SBE-Luc) in a pancreatic cancer cell line, PANC-1, they screened a library of approximately 16000 compounds from ChemBridge DIVERSet, and finally identified scriptaid compound, which increased Smad transcriptional activity.

It is well known that expression of TGF-β receptors plays an important role in TGF-β-mediated cell growth inhibition. Therefore, increasing expression levels of TGF-β receptors in cancer cell lines, in which TGF-β receptors are mutated or their expression levels are attenuated, may be a good therapeutic target in cancer treatment. In fact, it has been reported that HDAC inhibitors, such as MS-275 and suberoylanilide hydroxamic acid (SAHA), increase response to TGF-β by inducing the expression of TGF-β-RⅡ and TGF-β-RⅠ, respectively, in breast cancer cell lines[66,67]. Besides, it has been found that the expression level of TGF-β-RⅡ is increased by captopril (angiotensin-converting enzyme inhibitor), FTI-27742 (farnesyl transferase inhibitor), and 5-aza-2'-deoxycytidine (DNA methyltransferase inhibitor)[68-70]. Thus, these compounds may be used as anti-cancer drugs or for combination treatments when treating colon or small cell lung cancer that commonly displays decreased expression levels of TGF-β-RⅡ.

Intriguingly, the screened chemical compounds including A-161906 and scriptaid that were identified by high-throughput screening, diheteropeptin, and spiruchostatins are strong HDAC inhibitors. This indicates that the activity of HDAC may play a key role in the regulation of TGF-β signaling. Recently, the FDA has approved SAHA (Vorinostat® Zoniza) as a treatment for cutaneous T-cell lymphoma (CTCL). It has been reported that SAHA activates p21 and induces apoptosis of T cells in CTCL patients[71].

Many transport molecules of TGF-β signaling as well as TGF-β receptors are regulated by epigenetic mechanisms. Therefore, even though HDAC inhibitors do not induce selective activation during TGF-β signal transduction, it is considered that HDAC inhibitors and various chemical compounds similar to or increasing TGF-β activity can be good targets in the prevention and treatment of cancer. Since the most frequent genetic alteration found in cancer is the loss-of-function in tumor suppressor genes, this alteration is very specific to cancer cells and it may be a good therapeutic target in the development of anti-cancer drugs. In order to identify the agents selectively targeting cancer cells with loss-of-function mutations, Wang *et al*[72] have demonstrated a novel screening technique, named pharmacologic synthetic lethal screening, and have identified UA62001, which shows selectivity against DPC4-deficient cell line BxPC-3, and by investigation of

downstream target genes of UA62001, they have found that UA62001 arrests cells in G2/M phase by inhibiting cyclin B/CDC2 expression. In addition, Park *et al*[73] have screened the Maybridge Diversity Set chemical library using a cell-based assay to measure nuclear translocation of Smad proteins. Through high-throughput screening, DAM-1976, which enhances TGF-β signaling, has been identified. DAM-1976 increases sensitivity to TGF-β by prolonging Smad2/3 activation in the nucleus in a TGF-β-independent manner.

Professor Sporn MB, who discovered TGF-β and has been in the vanguard of cancer chemoprevention research, and his colleagues[74] have made many modifications of triterpenoids that possess anti-inflammatory and anti-tumor activity. They have screened derivatives of two known triterpenoids, oleanolic acid, and ursolic acid, and synthesized 2-cyano-3,12-dioxooleana-1, 9-dien-29-oic acid (CDDO), which has the most potent anti-inflammatory activity and low toxicity. It has been reported also that CDDO not only promotes Smad signal transduction, but also displays various biological activities similar to that of TGF-β[75]. Moreover, CDDO is currently in phase Ⅱ clinical trials for the treatment of leukemia and solid tumors, based on its anti-inflammatory and anti-tumor activities, cellular protection, and suppression of tumorigenesis. Therefore, CDDO is considered to be a promising chemopreventive and chemotherapeutic agent in cancer treatment.

TGF-β antibody and soluble TGF-β receptors

The monoclonal antibody that neutralizes TGF-β ligand is one of most developed inhibitors for the clinical application of suppressing TGF-β activity. The human monoclonal antibodies CAT-152 (lerdelimumab), CAT-192 (metelimumab) and GC1008, and the mouse monoclonal antibodies 1D11 and 2G7 against TGF-β have been generated to date. CAT-152 and CAT-192 are the human IgG4 monoclonal antibodies that neutralize TGF-β2 and TGF-β1, respectively. CAT-152 and CAT-192 (Cambridge Antibody Technology) have entered phase Ⅲ and Ⅱ clinical trials for the treatment of wounds after glaucoma filtration surgery and pachyderma, respectively. However, the trials were discontinued in 2004 due to problems concerning their effectiveness[76]. GC1008, developed by Genzyme Corporation, is a pan-TGF-β selective monoclonal antibody that works against all TGF-β isomers. Clinical trials for GC1008 were conducted in idiopathic pulmonary fibrosis patients. Also, it is currently in a clinical trial for the treatment of metastatic skin cancer and renal cancer. The anti-tumor effectiveness of 1D11 and 2G7 has been studied in various animal tumor models. According to the report of Arteaga *et al*[77], intraperitoneal injection of 2G7 suppresses not only tumor growth of MDA-MB-231 breast cancer cell lines, but also lung metastasis. Moreover, 2G7 partially inhibits angiogenesis by reducing the levels of vascular endothelial growth factor in blood plasma. On the other hand, suppression of TGF-β activity can be achieved by using macromolecular proteins such as soluble TGF-β receptors that are able to inhibit competitively interactions between the ligand and the receptor. In particular, soluble TGF-β-RⅡ has been extensively studied in preclinical tumor models. A soluble chimeric protein, composed of the extracellular domains of the TGF-β-RⅡ and the Fc portion of IgG1, has been shown to block TGF-β activity[78]. When tested in a transgenic mouse model that expressed a soluble TGF-β-RⅡ receptor: Fc fusion protein (Fc:TGF-β-RⅡ), metastatic capacity was suppressed without any increase of tumor growth, which indicated that this fusion protein selectively inhibited metastasis-associated TGF-β activity[79]. In addition to soluble TGF-β-RⅡ, soluble TGF-β-RⅢ also suppressed tumor growth and lung metastasis in an MDA-MB-231 xenograft tumor model by antagonizing the tumor-promoting activity of TGF-β[80].

TGF-β receptor kinase inhibitors

TGF-β receptor kinases are not only key molecules in intracellular TGF-β signal transduction, but potential therapeutic targets that can be easily regulated by small molecular compounds. Owing to the success of Gleevec®, many projects, focused on TGF-β receptor kinases, have been carried out along with large pharmaceutical companies. In particular, numerous studies concerning TGF-β-R Ⅰ have been performed to elucidate the domain structures, biochemical activity, and biological functions of TGF-β-R Ⅰ [81]. Thus, TGF-β-R Ⅰ serves as an attractive target for drug development. The development of protein kinase inhibitors can be achieved in several ways, such as target hopping, novel screening and virtual screening.

TGF-β-R Ⅰ kinase inhibitors with various structures have been developed by drug companies. Their various effects including anti-tumor activity confirmed in animal models, relations with TGF-β-induced Smad phosphorylation, activation of reporter genes, cell cycle arrest, and EMT, have been reported. SB-431542, SB-505124 (Glaxo Smith Kline; GSK), LY364947, LY2157299 (Eli Lilly), SD-208 (Scios), and IN-1130 (In2gen) are well known TGF-β-R Ⅰ kinase inhibitors. SB-431542 and SB-505124 compounds from GSK were developed by target hopping based on the structures of the kinases. Phosphorylation of TGF-β-R Ⅰ is inhibited by SB203580, a p38 kinase inhibitor, because TGF-β-R Ⅰ has a gatekeeper residue equivalent to that of p38 kinase, which results in structural similarity to p38 kinase[82]. SB-431542 was synthesized having SB-203580 as a lead compound. It has shown greater selectivity for TGF-β-R Ⅰ compared with other serine/threonine kinases (protein kinase C, extracellular signal-regulated kinase, JNK, p38 MAPK). However, it also has displayed strong inhibitory activity against activin receptor (ALK4) and nodal receptor (ALK7)[83]. In addition, SB-431542 inhibits proliferation of glioma, TGF-β-mediated morphological changes, and cell motility, which suggests that small molecule inhibitors of TGF-β receptor kinases have potential to suppress tumor progression[84].

Based on the growth inhibitory characteristics of TGF-β in epithelial cells, researchers at Eli Lilly (Indianapolis, MN, USA) have identified the heteroarylpyrazole structure of LY364947 through high-throughput screening[85]. After they confirmed that TGF-β-R I and TGF-β-R Ⅱ kinase were the molecular targets of LY364947, the latter became a lead compound in the development of inducers. LY364947 also turned out to be identical to HTS-466274, which was identified by Biogen Inc. through computer modeling of a compound database that contained over 200 000 compounds. The result suggested shape-based virtual screening as a powerful tool to discover and develop new TGF-β-R I kinase antagonists. Furthermore, studies on the relationship between structure and activation, as well as optimization of lead compounds have led to the development of more advanced compounds, such as LY2157299. In particular, LY2157299 entered a phase I clinical trial for the first time as a TGF-β-R I kinase antagonist. Also, studies on its pharmacotoxicity, pharmacokinetics, and pharmacodynamics are currently in progress[86].

Oral bioavailability of SD-208, another type of TGF-β-R I kinase inhibitor under development by Scios Inc., has been verified by demonstrating its ability to inhibit infiltration of glioma cells and metastasis of a highly metastatic breast cancer cell line[87]. Besides, A-83-01 has been found to be more potent in the inhibition of TGF-β-R I than SB-431542, and GW788388 can be administered orally, with improved pharmacokinetics[88,89]. IN-1130, recently developed in Korea as a small molecular inhibitor of TGF-β-R I , has shown kinase selectivity similar to that of SB-431542. IN-1130 also inhibits tumor growth and promotes activation of cytotoxic T cells in xenograft prostate tumor models, which suggests that it might restore TGF-β-induced immunosuppression in cancer cells[90].

Non-selective agents that suppress TGF-β signaling

Besides the selective inhibitory substances, including antibodies against the ligand and receptor kinase inhibitors, non-selective chemical compounds may provide strategies for the regulation of TGF-β signaling. Such agents include compounds that suppress the expression level of TGF-β, increase the expression level of Smad7, an endogenous inhibitory protein, or regulate the stability of TGF-β receptors (Figure 4B). Natural or synthesized agents that suppress TGF-β signaling through these mechanisms generally demonstrate anti-inflammatory, anti-fibrosis, and various biological activities. Among these, tranilast (Rizaben®) has been used as an anti-allergic drug for the treatment of asthma and atopy. Also, it has been known that tranilast has anti-fibrosis and anti-tumor effects. According to animal experiments recently carried out with the highly metastatic murine 4T1 breast cancer cell line, tranilast effectively suppressed metastasis by activating Smad proteins, inhibiting EMT, and strongly suppressing the production of TGF-β and IL-17 that were secreted by immune cells[91]. The suppressive effect of tranilast on metastasis turned out to be greater than that of TGF-β antibody 1D11 or paclitaxel.

In addition, pirfenidone, which is in a clinical trial for idiopathic pulmonary fibrosis, due to its anti-fibrosis effects, suppresses proliferation of neuroglioma by inhibiting expression and activation of TGF-β[92]. Halofuginone, a plant alkaloid and inducer of febrifugine, is a representative small molecular weight molecule in the suppression of collagen synthesis. It was first isolated from *Dichroa febrifuga*, approved by the FDA, and has been used for the treatment of scleroderma. Recently, clinical trials have been conducted on Kaposi's sarcoma to examine its inflammatory activity. Halofuginone has been reported to inhibit TGF-β signaling by decreasing TGF-β-R Ⅱ expression and elevating inhibitory Smad7 expression[93].

Moreover, tetrandrine, another plant alkaloid, and doxorubicin have demonstrated increased expression of Smad7[94,95]. While screening for agents that suppress TGF-β signal transduction, geldanamycin, which is also known as a heat shock protein 90 (Hsp90) inhibitor, was identified. Geldanamycin inhibits TGF-β-induced target gene expression and Smad phosphorylation even at the subnanomolar concentrations. Analysis of its mechanism of action revealed that geldanamycin suppresses TGF-β signaling *via* complex processes. For instance, geldanamycin regulates Hsp90 chaperone-associated stability of TGF-β receptors, suppresses receptor complex formation between TGF-β-R Ⅱ and TGF-β-R I , and induces interaction between Hsp70 and its receptors[96]. In general, Hsp90 inhibitors are widely recognized as a promising therapeutic target for anti-cancer drug development because of their ability to suppress tumor growth by inactivating various proteins that are related to tumorigenesis, such as Bcr-abl, ErbB2 and Raf-1. Various Hsp90 inhibitors, including geldanamycin inducer and 17-(allylamino)-17-demethoxygeldanamycin (17-AGG) are currently undergoing clinical trials. In fact, 17-AGG has completed a phase Ⅲ clinical trial for the treatment of multiple myeloma[97].

CONCLUSION AND FUTURE DIRECTIONS

Although TGF-β signaling has been shown to have some paradoxical actions in tumorigenesis, many factors activate TGF-β signaling for tumor suppression, such as HDAC inhibitor, SAHA, synthetic terpenoid, CDDO, in addition to inhibitors of TGF-β signaling. It is a good strategy to block the initiation of inflammation-associated tumorigenesis through the development of TGF-β mimics in order to achieve chemoprevention. Activators of TGF-β signaling inhibit initiation of tumorigenesis and invasion and metastasis of advanced-stage tumors. This sort of approach will be very helpful for TGF-β-responsive tumor therapy, so-called tumor vaccination. In terms of chemoprevention, the difficulty in applying TGF-β activators to normal people is due to immunosuppressive action and difficulty in detecting the tumor

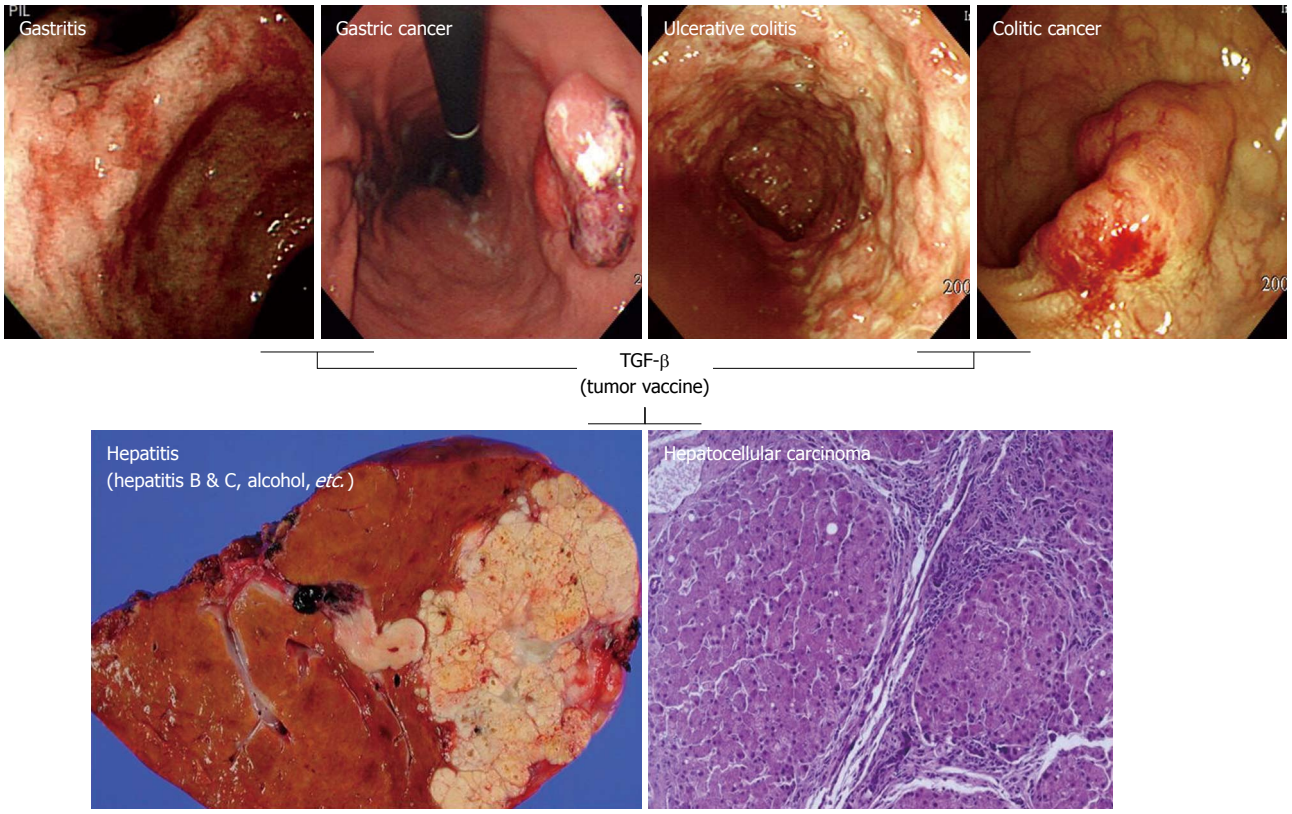


Figure 5 TGF-β1 as pivotal tumor vaccine based on anti-inflammatory actions that block progression from inflammatory lesions to precancerous lesions/cancer. Chronic inflammation induced by biological, chemical, microbial and physical factors is associated with increased risk of human cancer at various sites. Inflammation facilitates initiation of normal cell transformation, growth, and progression to malignancy through production of pro-inflammatory cytokines and diverse reactive oxygen species. In this pathway, TGF-β plays a key role in chemoprevention through anti-inflammatory and anti-mutagenic actions.

at an early stage. Blocking inflammation-associated carcinogenesis with the anti-inflammatory activity of TGF-β signaling can be used as a chemopreventive strategy in patients at high risk of cancer (Figure 5). Even though TGF-β may regulate the inflammatory process by modulating the differentiation of many kinds of T cells, we still do not know the exact role of TGF-β in T-cell biology. Further study of the role of TGF-β in development of GI tract immune cells can lead to future therapeutic approaches for inflammation and tumorigenesis. To date, tumor chemotherapy by suppression of TGF-β signaling has been tried, to enhance the tumor-specific immune reaction, inhibit angiogenesis of the tumor microenvironment, and block invasion and metastasis of tumor cells. However, TGF-β has different effects depending on the type or context of the tumor, and has an anti-inflammatory effect on various immune cells. For these reasons, it is important to select the right patients, time of intervention, therapeutic reagents to combine, and exact targets, to minimize the side effects and maximize successful treatment. Recently, many low molecular size inhibitors of TGF-β receptor kinase have been tested in clinical trials of tumor chemotherapy. Also, cross-talk between TGF-β signaling and other signaling pathways has been studied in detail, and new candidates have been screened and developed to regulate these signaling pathways. Based on results of cooperative activity of TGF-β with oncogenes, combination therapy with conventional anti-tumor reagents and TGF-β signaling inhibitors can enhance the therapeutic effects. Since inflammation-associated carcinogenesis is caused by complex cross-talk between many different cytokine signaling pathways, by collaboration with systems biologists, we can understand in more detail the mechanisms and networks of signaling pathways for inflammation and tumorigenesis. Through this approach, we can design and develop more efficient therapeutic drugs for treatment of GI cancer based on the biological action of TGF-β.

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Acute lung injury and ARDS in acute pancreatitis: Mechanisms and potential intervention

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Abstract

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) in acute pancreatitis still represents a substantial problem, with a mortality rate in the range of 30%-40%. The present review evaluates underlying pathophysiological mechanisms in both ALI and ARDS and potential clinical implications. Several mediators and pathophysiological pathways are involved during the different phases of ALI and ARDS. The initial exudative phase is characterized by diffuse alveolar damage, microvascular injury and influx of inflammatory cells. This phase is followed by a fibro-proliferative phase with lung repair, type Ⅱ pneumocyte hypoplasia and proliferation of fibroblasts. Proteases derived from polymorphonuclear neutrophils, various pro-inflammatory mediators, and phospholipases are all involved, among others. Contributing factors that promote pancreatitis-associated ALI may be found in the gut and mesenteric lymphatics. There is a lack of complete understanding of the underlying mechanisms, and by improving our knowledge, novel tools for prevention and intervention may be developed, thus contributing to improved outcome.

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Key words: Acute lung injury; Acute respiratory distress syndrome; Acute pancreatitis; Etiology; Pathophysiology

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INTRODUCTION

The incidence of acute pancreatitis is in the range of 300 or more patients per million annually[1,2]. Using the Atlanta classification on severity, about 10% of acute pancreatitis patients are classified as severe[3]. About one-third of all deaths from acute pancreatitis has been reported to occur prior to admission to hospital, and in most cases, is associated with acute lung injury (ALI)[4]. Hospital deaths occur within the first week after admission in 35%-50%[1,5,6] and the cause of death is related to single or multiple organ failure in a majority of cases[7]. In elderly patients, up to 60% of all deaths within the first week are considered to be caused by pancreatitis-

associated ALI and acute respiratory distress syndrome (ARDS)[8]. Independently, ALI is a consequence of a pronounced systemic inflammatory response with increased endothelial and epithelial barrier permeability, with leakage of a protein-rich exudate into the alveolar space and interstitial tissues, thus compromising oxygenation and gas exchange[9]. The magnitude of the systemic inflammatory response determines the concomitant clinical course and outcome[10,11] and this also is true for the severity of the acute-pancreatitis-associated ALI[12] (Figure 1). Respiratory complications are frequent in acute pancreatitis, and respiratory dysfunction, presenting as ALI or ARDS, is a major component of multiple organ dysfunction syndrome (MODS), with a frequent need for ventilatory support[8,13], which contributes to early death in severe acute pancreatitis[14] (Figure 2).

The mortality in ALI has been reported as 30%-60%, and is higher in elderly patients[15,16]. In those who survive, the quality of life is impaired[17]. Overall, ALI and ARDS represent the most common and earliest organ dysfunction in the development of MODS, in which mortality is related to the number of involved organs[18]. This type of secondary ALI, a dominant part of MODS, is also found in severe acute pancreatitis, in which lung injury has been reported to account for a high percentage of deaths.

Acute respiratory failure, including ALI and the more severe form, ARDS, has radiological findings with bilateral pulmonary infiltrates and physiological changes, normal cardiac filling pressures, and a ratio of arterial oxygen pressure and inspiratory oxygen concentration (PaO_2/FiO_2 < 300 mmHg for ALI and < 200 mmHg for ARDS, which reflects pronounced morphological changes)[19]. ALI and ARDS frequently occur in critically ill patients, although the exact incidence in acute pancreatitis has not been stated. If we extrapolate Scandinavian data on ALI and ARDS patients, mortality in the United States is about 36 000 patients per year[20]. More recent mortality rates have also been reported to be 30%-40% and higher in elderly patients[21].

MECHANISMS

Two different phases in ALI and ARDS have been described. Initially, an exudative phase during the first days with diffuse alveolar damage, microvascular injury, type I pneumocyte necrosis, and influx of inflammatory cells and fluid to the pulmonary interstitium has been seen, followed by a fibro-proliferative phase during days 3-7, during which type II pneumocyte hyperplasia, proliferation of fibroblasts and lung repair occur[22]. As a consequence of a pronounced and complex systemic net pro-inflammatory response, both endothelial and epithelial injury is involved in ALI and ARDS (Figure 3). Mediators are several, including cytokines and chemokines, pro-inflammatory mediators and a variety of cells, which regulate the migration and pulmonary infiltration of neutrophils into the interstitial tissue, where they cause injury and breakdown of the pulmonary parenchyma[23]. In order to give a sense of the significance of acute pancreatitis as an etiological factor for ARDS in ICU patients,

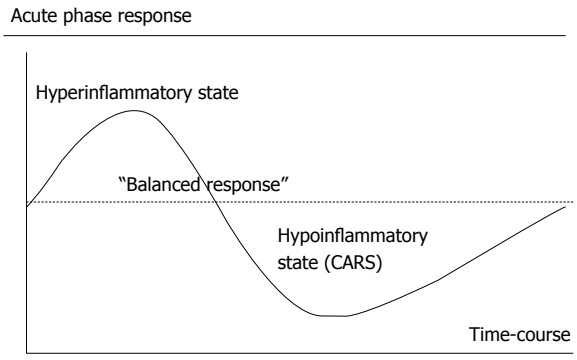


Figure 1 The acute phase response as seen in critical illness, e.g. severe acute pancreatitis.

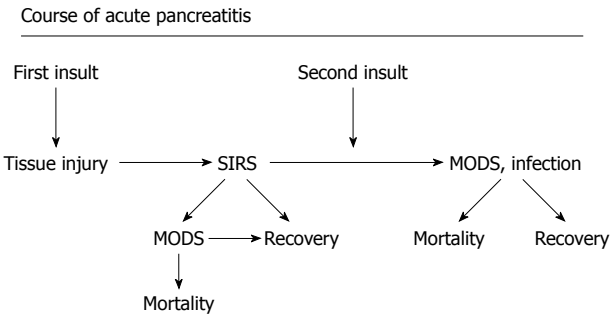


Figure 2 Course of acute pancreatitis. A potential development in severe acute pancreatitis with the first "insult" resulting in a pronounce systemic inflammatory response and potential development of organ dysfunction, and in the worst scenario early mortality. Later during the course, combination of organ dysfunction and infection, potentially pronounced after the second "insult" (translocation from the gut, burst of proinflammatory cytokines, surgery, *etc.*) may result in late mortality. MODS: Multiple organ dysfunction syndrome.

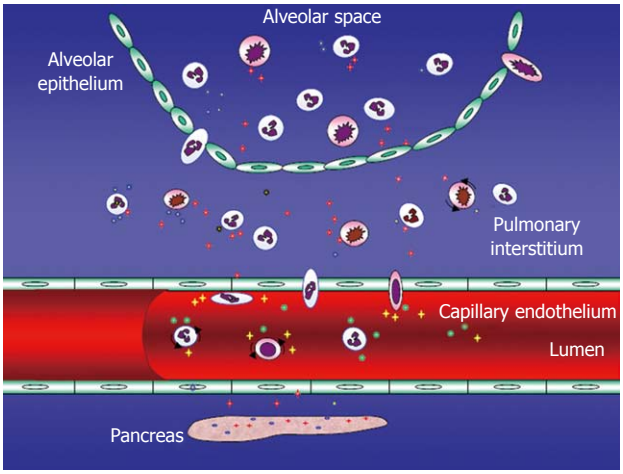


Figure 3 Acute pancreatitis-associated acute lung injury (ALI) - potential mechanisms including endothelial barrier dysfunction. Several adhesion molecules [selectins, intercellular adhesion molecule-1 (ICAM-1), platelet endothelial cell adhesion molecule-1 (PECAM-1) among others] involved in the extravasation of not at least polymorphonuclear neutrophils (PMNs). Tissue injury by not at least these PMNs.

almost one out of seven patients have acute pancreatitis as a primary cause[24].

PATHOPHYSIOLOGICAL MECHANISMS IN SECONDARY ALI

ALI and ARDS may occur secondary to acute pancreatitis, with similar appearances. In general, controlling the source of what is actually fuelling the ALI is important. The features of secondary ALI/ARDS thus involve an initial exudative phase with diffuse alveolar damage, microvascular injury, type- I pneumocyte necrosis and influx of inflammatory cells, followed by a fibro-proliferative phase with lung repair and type- Ⅱ pneumocyte hyperplasia and proliferation of fibroblasts[13]. Both endothelial and epithelial injury is involved. These changes in ALI, which involve endothelial barrier dysfunction, neutrophil and monocyte/macrophage activation, adhesion molecule expression and intracellular signaling, can to a great extent be executed by proteases derived from polymorphonuclear neutrophils (PMNs), and the process seems driven by tumor necrosis factor (TNF)-α and monocyte chemoattractant protein (MCP)-1, with involvement of mast cells, at least during the initiation of leukocyte activation[25-27]. These complex mechanisms that underlie the ALI associated with acute pancreatitis, and the variety of cells involved, which contribute to neutrophil recruitment, adhesion and activation, as well as signal transduction pathways such as tyrosine kinase activation, local transcription of nuclear factor-κB, and expression of multiple inflammatory genes, have been described in a number of experimental studies and reviews[9,13,28,29]. It thus seems well established that inflammatory mediators play a key role in the pathogenesis of ALI and ARDS. These mediators include TNF-α, interleukins-1β, -6, and -10, transforming growth factor-β, granulocyte-macrophage colony-stimulating factor, platelet-activating factor (PAF), selectin and adhesion molecules, complement component C5a, neuropeptide substance P, and chemokines such as MCP-1, and macrophage inflammatory protein-1α. Moreover, one of the results seems to be the production of reactive oxygen and nitrogen species with potential deleterious effects on pulmonary endothelial and epithelial functions[12,29,30]. The neuropeptide substance P possesses pro-inflammatory action that increases vascular permeability, evidently acting through neurokinin-1 receptors. The complement component C5a is a pro-inflammatory chemoattractant that, at least in the experimental setting, seems to increase lung injury, as does the CD40 receptor found on lymphocytes, monocytes and dendritic cells[30]. All of these represent potential targets for future intervention. It is thus the net magnitude of the pro-inflammatory response and the ratio between pro- and anti-inflammatory activity during the process that ultimately determine the outcome of pancreatitis-associated ALI/ARDS. The means of activating leukocytes may be complex and different, and also involve mast cells during the initiation of leukocyte activation. Pulmonary macrophages are likely to be involved in pancreatitis-induced endothelial barrier dysfunction, compromise of type Ⅱ pneumocytes, and tissue injury, and furthermore, the release of matrix metalloproteinases seems essential, not at least, that derived from mast cells[31,32].

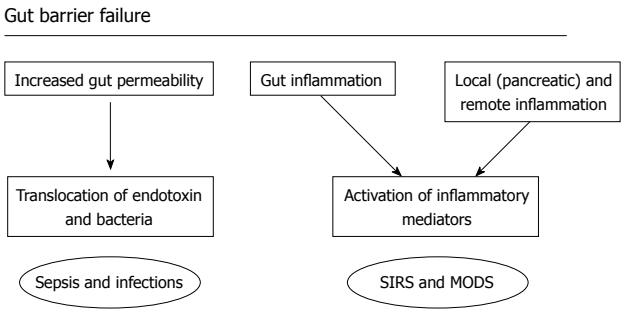


Figure 4 Gut barrier failure. The increase in permeability of the gut barrier from the intestinal lumen may allow translocation of endotoxin and bacteria and there is also activation of immunocompetent cells in the gut wall and gut associated lymphoid tissue, contributing to the inflammatory response, infection, and potentially the development of organ dysfunction.

Pancreatitis-associated ALI has been reported to be related to the effects of pancreatic enzymes, and in particular, phospholipase A_2 is thought to play a role in ALI by damaging pulmonary surfactant, which is a substrate for phospholipase A_2[33]. Furthermore, patients with severe acute pancreatitis have been found to have elevated serum concentrations of phospholipase A_2, which are correlated with the extent of pulmonary complications[34], and a correlation between lung injury score and serum concentration of phospholipase A_2 has been identified[35]. It should be mentioned that the increased systemic levels of phospolipase A_2 that have been reported in this study may be derived not only from the pancreas, but also be of non-pancreatic origin[35]. With all these mechanisms involved, including a variety of different mediators and cells, there have been high expectations on the use of various types of pancreatic protease inhibitors.

DOES THE GUT PLAY A ROLE IN ALI?

The gut barrier not only represents a mechanical line of defense, i.e. a border between the gut lumen and its contents and the rest of the body, but also includes a variety of immunocompetent cells both within the intestinal wall and in associated lymph nodes, which are represented as mucosa-associated lymphoid tissue and gut-associated lymphoid tissue. Preserving an intact gut barrier is thus a complex and meticulous process that is impaired in critical illness such as severe acute pancreatitis, in which the compromised barrier allows for increased intestinal permeability and translocation of bacteria derived from the intestinal lumen and toxins, as well as a gut inflammatory state[10,36-39] (Figure 4).

Mesenteric lymphatics have been found to carry gut-derived factors that contribute to ALI in various

experimental models[40-42]. Interruption of the flow of mesenteric lymph leads to amelioration of ALI, but still we have not identified the exact factors derived from the gastrointestinal tract that are responsible for the pulmonary and systemic effects. This observation represents one potential tool to achieve control of the systemic response and decrease pulmonary injury by modulating factors derived from the gut[43,44].

OUTCOME OF EXPERIMENTAL STUDIES AND POTENTIAL CLINICAL IMPLICATIONS

Our improved understanding of the underlying pathophysiological mechanisms involved in ALI in critical illness has led to a corresponding expectation about potential clinical interventions. This concerns the role of the inflammatory response and signaling mechanisms, such as the protein kinase C pathway[30-32]. Pretreatment and early treatment in experimental acute pancreatitis with, for example, a PAF antagonist and monoclonal antibodies against adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and platelet endothelial cell adhesion molecule-1 (PECAM-1) have been successful[26,27,45]. When evaluating clinical trials with a variety of non-antibiotic interventions in acute pancreatitis, outcome has been less favorable with contradictory results for octreotide and its analogs, as well as the use of the intracellular protease inhibitor gabexate[46]. High expectations have been raised for the use of the highly specific PAF antagonist lexipafant, which has been shown to reduce organ failure and the inflammatory response in patients with predicted severe acute pancreatitis, when administered early[47,48]. A concomitant major study was less convincing, although it did report decreased organ failure inflammatory mediators[49].

FUTURE ASPECTS

Cross-talk between coagulation and inflammation evidently seems to exist, as exemplified by treatment with recombinant human activated protein C in patients with severe acute pancreatitis, in whom a reduction in mortality has been reported[50]. Other components of the coagulation cascade seem to possess inflammatory properties to various degrees. For example, blockers of tissue factor or factor VIIa in experimental severe acute pancreatitis have been shown to ameliorate the associated ALI and decrease neutrophil influx, both when administered as pretreatment and as early treatment[51]. The role of anticoagulants as anti-inflammatory agents in ALI may represent a novel therapeutic option and should be further investigated[52].

The epithelium is involved early in the development of ALI, and produces pro-inflammatory chemokines and triggers neutrophil migration. Furthermore, the epithelium interacts with pulmonary macrophages, which may exacerbate production of pro-inflammatory mediators, thereby increasing recruitment of PMNs from the circulation to the pulmonary interstitial tissue and alveolar lumen. The blocking of chemokines, for example, MCP-1, may thus represent an interesting mode of intervention[53].

Gram-negative infections may be an important predisposing factor for ARDS in acute pancreatitis and endotoxin might potentiate ALI[54]. This emphasizes translocation from the gastrointestinal tract to the systemic circulation and remote organs, as well as the role of the gut-lymph-lung axis. Toll-like receptor 4 (TLR4) compromises the innate immune response and initiates complex signaling pathways when interacting with lipopolysaccharide, which ultimately results in a pro-inflammatory response. Amelioration of the severity of acute pancreatitis and reduced lung injury has been noted in mice that lack TLR4[55], and the lung injury decreases in severity in experimental severe acute pancreatitis treated with nitric oxide, which affects TLR4 gene expression[56]. Therefore, TLR4 has been emphasized as a potential future therapeutic target against inflammatory processes[57].

Heparan sulfate derived from the extracellular matrix or the surface of epithelial cells induces a pro-inflammatory response and has, in its soluble form, been suggested to activate TLR4, thus triggering an endogenous pathway to initiate cytokine production[58]. In experimental acute pancreatitis, heparan sulfate aggravates pancreatic inflammation, and promotes the progression from a local to a systemic inflammatory response, which implies that blocking of heparan sulfate is a potential route of intervention[59]. It has also been suggested that heparan sulfate is important during the initiation of acute pancreatitis, by aggravating the local inflammatory response within the pancreas and thereby potentially promoting the development of a more pronounced systemic inflammatory response, in which heparan sulfate is cleaved off from the epithelial lining of the pancreatic ducts[60]. The role of heparan sulfate and its inhibition thus represents an interesting future line of research, both for the initiation of acute pancreatitis, as well as the development of a systemic inflammatory response.

CONCLUSION

ALI and ARDS in acute pancreatitis remains a major challenge that requires substantial resources. Presently, there is a lack of interventions directed at the underlying pathophysiological mechanisms, although improved understanding may provide us with novel tools for prevention and intervention. Ongoing research on acute pancreatitis and acute respiratory injury gives hope of improvement in the management of this severe and resource-demanding complication, that is still associated with substantial mortality.

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ORIGINAL ARTICLE

Inhibition of hepatitis C virus replication by single-stranded RNA structural mimics

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Abstract

AIM: To examine the effect of hepatitis C virus (HCV) structural mimics of regulatory regions of the genome on HCV replication.

METHODS: HCV RNA structural mimics were constructed and tested in a HCV genotype 1b aBB7 replicon, and a Japanese fulminant hepatitis-1 (JFH-1) HCV genotype 2a infection model. All sequences were computer-predicted to adopt stem-loop structures identical to the corresponding elements in full-length viral RNA. Huh7.5 cells bearing the BB7 replicon or infected with JFH-1 virus were transfected with expression vectors generating HCV mimics and controls. Cellular HCV RNA and protein levels were quantified by real-time polymerase chain reaction and Western blotting, respectively. To evaluate possible antisense effects, complementary RNAs spanning a mimic were prepared.

RESULTS: In the BB7 genotype 1b replicon system, mimics of the polymerase (NS-5B), X and BA regions inhibited replication by more than 90%, 50%, and 60%, respectively. In the JFH-1 genotype 2 infection system, mimics that were only 74% and 46% identical in sequence relative to the corresponding region in JFH-1 inhibited HCV replication by 91.5% and 91.2%, respectively, as effectively as a mimic with complete identity to HCV genotype 2a. The inhibitory effects were confirmed by NS3 protein levels. Antisense RNA molecules spanning the 74% identical mimic had no significant effects.

CONCLUSION: HCV RNA structural mimics can inhibit HCV RNA replication in replicon and infectious HCV systems and do so independent of close sequence identity with the target.

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Key words: Hepatitis C virus; Japanese fulminant hepatitis virus; Complementarity; RNA sequence; Hybridization

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Smolic R, Smolic M, Andorfer JH, Wu CH, Smith RM, Wu GY. Inhibition of hepatitis C virus replication by single-stranded RNA structural mimics. *World J Gastroenterol* 2010; 16(17): 2100-2108 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i17/2100.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i17.2100

INTRODUCTION

Chronic hepatitis C virus (HCV) infection is a leading cause of chronic hepatitis, liver cirrhosis and hepatocellular

carcinoma[1-3]. Current therapeutic options are limited and associated with significant adverse effects[4,5]. Accordingly, there is a strong impetus to develop novel therapeutic options. Because HCV replicates through the interaction of its RNA-dependent RNA polymerase and its RNA template to generate progeny RNA, physical contact between the enzyme and genomic RNA is required[6-8]. We hypothesized that mimics could be created with sufficient resemblance to the natural genomic structure to compete with the HCV genome, resulting in inhibition of replication. HCV RNA has a number of cis-acting replication elements (CREs) whose function could potentially be inhibited by structural RNA mimics[9]. For example, structural mimics based on the HCV internal ribosome entry site (IRES) were recently shown to inhibit HCV translation *in vitro* and in replicon cell culture[10]. Synthesis of (+)-strand RNA molecules was inhibited by RNA aptamers specific for the (-)-strand IRES domain corresponding to the 3'-terminal end of the negative strand of HCV RNA[11,12]. In addition to the IRES[13-15], HCV RNA bears CREs in the positive-strand NS5B[6,8,16,17] and X region[18,19] as well as in the negative strand 3'-terminal region[13,20]. The function of each CRE is assumed to depend on the ability of these structures to bind host factors[21-24] and viral non-structural proteins[8,14,15,25,26]. The objective of the current study was to determine to what extent computer-predicted single-stranded HCV RNA structures could inhibit HCV replication.

MATERIALS AND METHODS

Cloning of HCV RNA structural mimics

For replicon studies, structural mimics were constructed based on RNA sequences of the HCV 1b subgenomic replicon, BB7[27,28] (Table 1). Table 1 shows the regions and the nucleotides from which the mimic sequences were derived. Mimics 5B and X were designed to be identical to the (+) strand of the NS5B and X regions (Figure 1A and D, respectively). Mimics BA (Figure 1B) and EC (Figure 1C) were designed to be identical to the (-) strand of the 3'-terminus. The HCV RNA structural mimic sequences were predicted to adopt stem-loop structures identical to the corresponding CRE in full-length viral RNA, as determined by the Mfold ver 3.2 web server[29,30]. For Japanese fulminant hepatitis virus (JFH-1) infection studies, mimic sequence identities corresponding to HCV JFH-1 genotype 2a were designed to vary from 100% (5B-100), 74% (5B-74) and 46% (5B-46), the latter achieved by changes in the stem regions. The 5B-74 mimic was identical to the mimic (NS5B) used in genotype 1b replicon studies. To construct 5B-46, base-pair exchanges were made at positions 2-15, 18, 30-43, 49-53, 57-60, 75-78, 89-94 in 5B-74 (Figure 2). Regions in the stems or loops in which changes were predicted by Mfold ver 3.2 to alter the secondary structure were left intact. DNA fragments containing each mimic sequence flanked by *Bam*H I and *Hin*dⅢ sites were generated by polymerase chain reaction (PCR) amplification of sequences from pHCV rep1bBB7 (Apath, LLC, St. Louis, MO, USA). As a negative control, an unrelated [hepatitis B virus (HBV) encapsidation signal] was amplified by PCR from plasmid adwR9 (from Dr. T Jake Liang, NIH, Bethesda, MD, USA). The region cloned included HBV sequences flanking a 60 nucleotide (nt) element, so that the total insert length approximated that of the HCV RNA structural mimics. Each insert was cloned into a T7 transcription vector, pENT7 by *Bam*H I /*Hin*dⅢ digestion and ligation. pENT7 is a derivative of pENTR4 (Invitrogen Co., Carlsbad, CA), in which a T7 expression cassette (*Bam*H I and *Hin*dⅢ sites downstream of the consensus T7 RNA polymerase promoter) had been inserted in the multiple cloning site.

For expression of HCV RNA structural mimics as polymerase Ⅱ transcripts in mammalian cells, each insert was subcloned into pSilencer 4.1-CMV puro (Ambion) by *Bam*H I /*Hin*dⅢ digestion and ligation. To confirm fidelity, clones containing pSilencer 4.1-CMV puro with HCV RNA structural mimics were sequenced with primers as recommended by the manufacturer: 5'-AGGCGATTAAGTTGGGTA-3', 5'-CGGTAGGCGTGTACGGTG-3'.

Cloning of a 5B-100 structural mimic

As a positive control for HCV JFH-1 genotype 2a inhibition, a 5B-100 mimic was constructed to be 100% identical to the HCV JFH-1 genotype 2a NS5B coding region (Table 1). This mimic was also predicted using web server Mfold ver. 3.2 to adopt stem-loop structures identical to the corresponding CRE in full-length viral RNA.

Cells

Huh7.5 cells, a human hepatoma cell line, bearing the BB7 HCV genotype 1b replicon were obtained from Apath (St. Louis, MO). Huh7.5 cells, which support JFH-1 replication, were obtained from Dr. Charles M. Rice, Rockefeller University, NY, USA[16,31,32]. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1X antibiotic/antimycotic solution (Invitrogen). Cells were passaged every 3-4 d to maintain 75% confluence.

Transfection of plasmids for in situ generation of HCV structural mimics

Huh-7.5 cells containing the BB7 replicon, or cells carrying persistent infection with JFH-1 HCV RNA were seeded at a density of 10^5 cells in 2 mL of growth medium per well in 6-well plates, 6 d before transfection. For transfection, 25 μg of each plasmid encoding a structural mimic were transfected into Huh7.5 cells, 95% confluent, with 15 μL Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Plasmid DNA was diluted in 250 μL of Opti-MEM I Reduced Serum Medium (Invitrogen) without serum. Lipofectamine 2000 was diluted to the appropriate amount in 250 μL of Opti-MEM I medium. After 5 min incubation, diluted

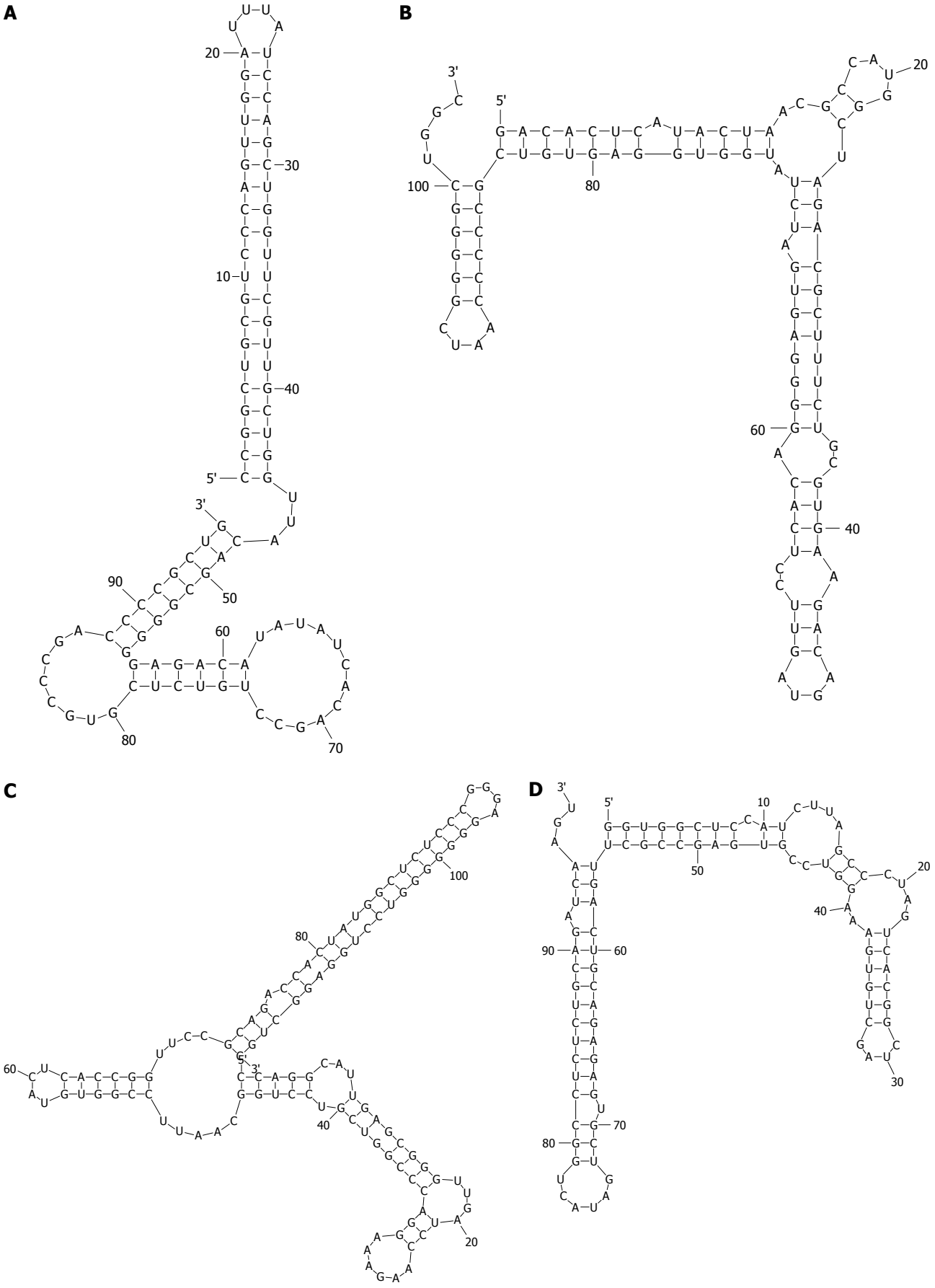


Figure 1 Predicted folding of RNA sequences designed as mimics of hepatitis C virus (HCV) genotype 1b regions. HCV RNA sequences were predicted by Mfold ver 3.2 to adopt stem-loop structures identical to the corresponding sequences in full-length viral RNA. A: 5B-74 corresponding to NS-5B (+) strand; B: BA region 3'-terminus of (-) strand; C: EC region 3'-terminus of (-) strand; D: X region of (+) strand.

Table 1 HCV RNA structural mimics

Structural analogs	Source BB7 sequence (nt)	Source JFH sequence (nt)	Replicon strand	JFH strand	Stem-loop domains
NS-5B (5B-74)	9216-9310		(+) strand		NS5B coding region
X	9508-9605		(+) strand		X region
EC	107-222		(-) strand		3'-terminus
BA	1-104		(-) strand		3'-terminus
5B-100		9284-9378		(+) strand	NS5B coding region

HCV: Hepatitis C virus; JFH: Japanese fulminant hepatitis.

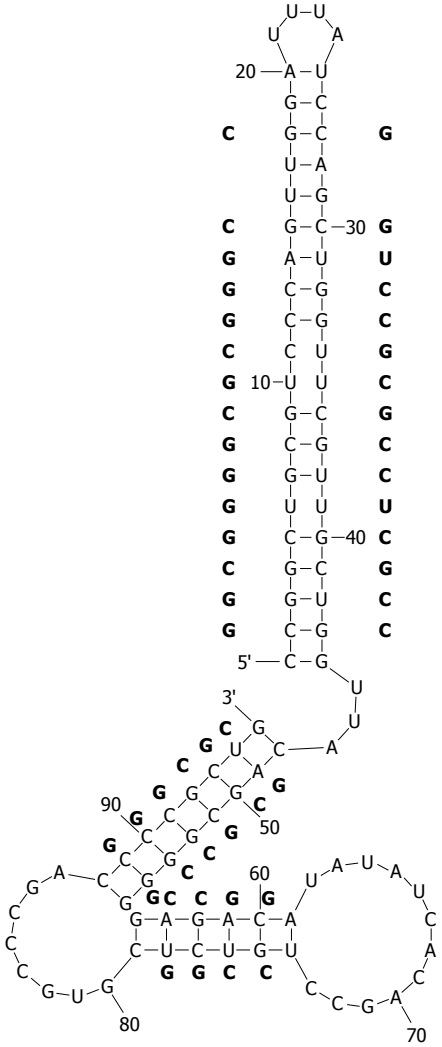


Figure 2 Diagram showing the nucleotide replacements (in bold) in 5B-74 made to create the 5B-46 structural mimic and predicted stem-loop structures identical to the corresponding sequences in 5B-74.

DNA was combined with diluted Lipofectamine 2000, mixed and incubated for 20 min at 25℃. Complexes in 500 μL medium were added. After 6 h of incubation at 37℃ under 50 mL/L CO_2, 1.5 mL of DMEM with 15% FBS were added to each well, and cells were incubated at 37℃ under CO_2 for 48 and 72 h. Cells were harvested and lysates assayed for NS3 protein levels by Western blotting. Total RNA was isolated and HCV RNA levels were determined by real-time reverse transcriptase PCR (RT-PCR).

HCV infection system

A cDNA from hepatitis C genotype 2a virus, JFH-1 strain (from Dr. Takaji Wakita, National Institute of Infectious Diseases, Tokyo, Japan)[33] has been shown to replicate autonomously without drug selection or adaptive mutations[34-37]. This cDNA was used to transfect the full-length JFH-1 genome into Huh7.5 cells to produce infectious HCV[38].

Real-time RT-PCR

RNA was isolated from BB7 replicon cells with Trizol (Invitrogen), and treated with RNase-free DNase (Promega). One microgram of DNase-treated total RNA was reverse transcribed using an iScript cDNA Synthesis Kit (BioRad Laboratories, Hercules, CA, USA). After incubation at 25℃ for 5 min, at 42℃ for 30 min and at 85℃ for 5 min, the resulting cDNA was quantified by real-time RT-PCR with SYBR GREEN according to the manufacturer's protocol (Roche Applied Science, Indianapolis, IN, USA) using HCV genotype 1b specific primers: forward primer: 5'-CTGTCTTCACGCAGAAAGCG-3' and reverse primer: 5'-CACTCGCAAGCACCCTATCA-3'. *Homo sapiens* lactate dehydrogenase A (LDHA) mRNA levels in each sample were simultaneously quantified to normalize values of HCV RNA. The primer sequence for LDHA forward primer was: 5'-TAATGAAGGACTTGGCAGATGAACT-3' and reverse primer: 5'-ACGGCTTTCTCCCTCTTGCT-3'. Assays were performed in triplicate and results expressed as mean ± SD of HCV replication as a percent of unrelated control.

For the JFH infection system, RNA replication in transfected cells was quantified by RT-PCR using HCV genotype 2a specific forward primer: 5'-TAGGAGGGCCCATGTTCAAC-3', reverse primer 5'-CCCCTGGCTTTCTGAGATGAC-3'. The PCR conditions were: 2 min at 50℃, 10 min at 95℃ and 15 s at 95℃. After 40 cycles, final extension was performed at 60℃ for 1 min. Assays were performed in triplicate and results expressed as mean ± SD of HCV replication as a percent of unrelated control.

Western blotting analyses

Total protein lysates of Huh7.5 cells stably infected with JFH-1 were evaluated by Western blotting. Cells were harvested in RIPA buffer (50 mmol/L Tris-HCl, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA) supplemented with protease inhibitors

(Roche). Protein concentrations were determined with Bio-Rad assay as described by the manufacturer. Forty micrograms of protein was resolved by SDS-PAGE, and transferred to Hybond nitrocellulose membranes (Amersham Pharmacia, Piscataway, NJ, USA). Membranes were blocked with 5% nonfat milk in PBS, incubated with a 1:1000 dilution of the monoclonal mouse HCV NS3 antibody (Biodesign International, Saco, ME, USA), washed with PBS/0.05% Tween 20 and incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (Pierce, Rockford, IL, USA) at 1:20 000 dilution. Bound antibodies were detected with SuperSignal chemiluminescent substrate (Pierce) and quantified by Gel Imaging software GeneTools (SynGene, Frederick, MD, USA). To ensure comparable loading of samples, blots were incubated with a 1:1000 dilution of a polyclonal rabbit α-tubulin antibody (Abcam Inc., Cambridge, MA, USA), and detected by HRP-conjugated goat anti-rabbit secondary antibody using procedures as described above.

Evaluation of possible antisense effects

Because the 5B-74 structural mimic was single-stranded, and retained significant (74%) sequence identity to JFH-1 genotype 2a sequence, it was possible that the observed inhibition of replication was a result of antisense effects. To assess this possibility, 4 complementary RNAs 21-28 nt long, spanning the 5B-74 mimic were synthesized (IDT-Integrated DNA Technologies, Inc., Coralville, IA, USA). The antisense sequences were: 5B-C1 (nt 5-21), 5'-CCGGCUGCGUCCCAGUUGGAU-3', 5B-C2 (nt 22-43), 5'-UUAUCCAGCUGGUUCGUUGCUG-3', 5B-C3 (nt 44-71), 5'-GUUACAGCGGGGGAGACAUAUAUCACAG-3' and 5B-C4 (nt 72-95), 5'-CCUGUCUCGUGCCCGACCCCGCUG-3'.

One day before transfection, JFH-1-infected Huh7.5 cells were plated in growth medium without antibiotics, to be 95% confluent at transfection. Oligomer-Lipofectamine 2000 complexes were prepared by dilution of 50 pmol or 500 pmol RNA oligomer in 250 μL of Opti-MEM I without serum. Five microliters of Lipofectamine 2000 was diluted in 250 μL Opti-MEM mixed gently and incubated for 5 min at 25℃. The diluted oligomer was combined with diluted Lipofectamine 2000, mixed and incubated for 20 min at 25℃. Complexes were added to each well containing cells and medium. After 6 h, 1.5 mL of 15% FBS diluted in DMEM was added and the cells were incubated at 37℃ in a CO_2 incubator for 48 h. HCV RNA replication was determined by real-time RT-PCR using HCV specific primers. Assays were performed in triplicate and results expressed as mean ± SD of HCV replication as a percent of unrelated control.

RESULTS

For replicon studies, structural RNA mimics were constructed based on sequences of the HCV 1b subgenomic replicon, BB7. Because HCV RNA replication involves generation of both positive and negative strands, structural mimics were designed for both strands. These targets were selected based on previous reports that HCV RNA bears CREs in the positive-strand NS5B coding region[6,8,16,17] and X region[18,19] as well as in the negative strand 3'-terminal region[13,39].

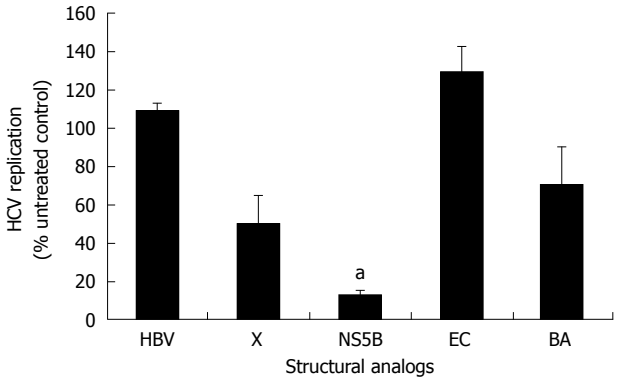


Figure 3 HCV RNA levels in the BB7 replicon cell lysates at 48 h post-transfection with structural mimics. BB7 replicon cells were transfected with plasmids generating structural mimics, and HCV RNA levels were assayed by real-time reverse transcriptase polymerase chain reaction (RT-PCR). The columns and bars represent the mean ± SD, respectively of 3 independent triplicate transfections. Statistically significant differences relative to controls are indicated by ($^aP < 0.05$).

The results of transfection of RNA mimics into BB7 replicon cells quantified by real time RT-PCR are shown in Figure 3. The 5B mimic inhibited HCV replication by more than 90%. Mimics to X and BA regions decreased replication by 50%, and 60%, respectively. The EC mimic actually had no inhibitory effect, and in fact appeared to increase replication, but the effect was not statistically significant. The fact that this sequence, also identical to the natural HCV sequence, failed to inhibit replication indicates that the observed inhibition by the NS5B mimic did not result from a nonspecific effect of HCV RNA. The inhibition by the NS5B plasmid was found to be dose-dependent up to 25 μg (data not shown).

Because the NS5B structural mimic appeared to be the most effective, further experiments were performed to determine whether this mimic might be effective against a different genotype in JFH-1-infected Huh-7.5 cells. It was found that the NS5B, now named 5B-74, mimic which was only 74% identical to the genotype 2a sequences of NS5B was as effective in suppression of HCV replication of JFH-1 genotype 2a as 5B-100 which was 100% identical to the genotype 2a (Figure 4).

The intent of the design of the structural mimics was to create single-stranded versions of the natural structure to compete for protein interactions. However, it is possible that the observed inhibitory effects could have been simply the result of non-specific effects. To explore this possibility, the mimic 5B-46 was prepared in which bases were changed at positions 2-15, 18, 30-43, 49-54, 57-60, 75-78, and 89-94 (Figure 2), with a sequence identity of the stems of only 27.5%, and overall

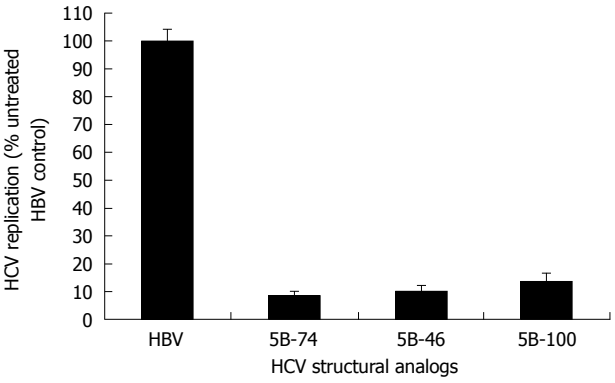


Figure 4 Effects of transfection of NS5B mimics 5B-74, 5B-46 and 5B-100 on Japanese fulminant hepatitis-1 (JFH-1) genotype 2a viral infection in Huh7.5 cells. Cells stably infected with JFH-1 genotype 2a virus were transfected with plasmids generating structural mimics, and HCV RNA levels were assayed by real-time RT-PCR. Assays were performed in triplicate and results expressed as mean ± SD of HCV replication percents of unrelated hepatitis B virus (HBV) control.

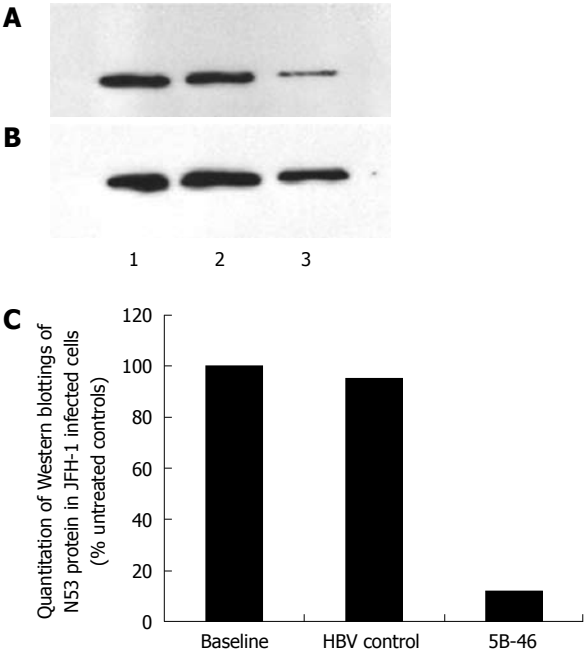


Figure 5 Western blotting analysis of NS3 protease gene expression in JFH-1 infected Huh7.5 cells after transfection of 5B-74 mimic and controls. A: HCV NS3; B: Tubulin, 72 h after transfection. Lane 1: Levels prior to addition of mimic; Lane 2: HBV (unrelated) control; Lane 3: 5B-46 mimic; C: Quantification of Western blottings of NS3 protein in JFH-1-infected cells at baseline, after 72 h exposure to 5B-46, and to HBV unrelated control. Bound NS3 antibodies were detected with SuperSignal chemiluminescent substrate and quantitated by Gel Imaging software GeneTools.

identity of 46%, relative to JFH-1 (genotype 2a) NS5B. The 5B-74 mimic decreased HCV replication by 91%. Furthermore, in spite of a sequence identity of only 46%, the novel mimic 5B-46, decreased HCV replication in JFH-1-infected cells by 90%, not significantly different from the mimic 5B-74 with 74% identity, or the mimic 5B-100 with complete identity to the HCV genotype 2a (Figure 4).

To assess viral protein levels, Western blottings of

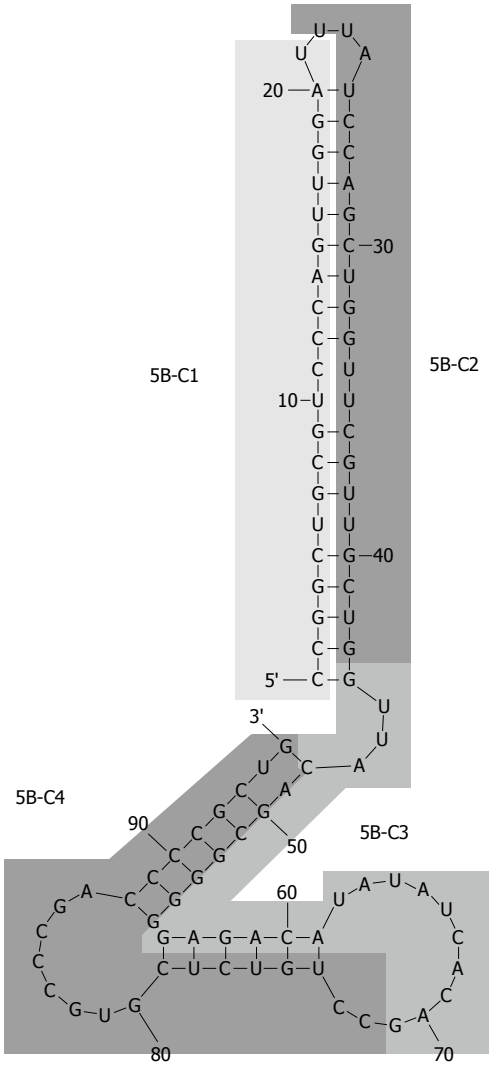


Figure 6 Diagram of single-stranded fragments spanning the entire length of the 5B-74 mimic used as antisense controls.

the NS3 protease were performed. Levels of neither the housekeeping protein tubulin (lane 2, Figure 5B) nor the NS3 protease (lane 2, Figure 5A) were affected by the unrelated HBV control mimic. However, the 5B-74 mimic decreased NS3 protease levels by 90% (lane 3, Figure 5A), while tubulin levels remained unchanged (lane 3, Figure 5B), the latter arguing against a non-specific effect or toxicity by the mimics. Quantification of the Western blottings confirmed the strong inhibitory effects of the 5B-46 mimic as shown in Figure 5C.

In the 5B-46 mimic, although most of the stem regions were altered, the sequences in the loops were left unchanged in order to retain secondary structure. Therefore, an antisense effect of the loop regions was still possible. To evaluate this possibility, short single-stranded fragments were prepared spanning the 5B-74 mimic (Figure 6). Analysis by Mfold ver 3.2 did not generate any stem-loop structures corresponding to regions in the native 5B-74 (data not shown). None of these sequences had any significant effects on HCV RNA levels compared to control (Figure 7), supporting the

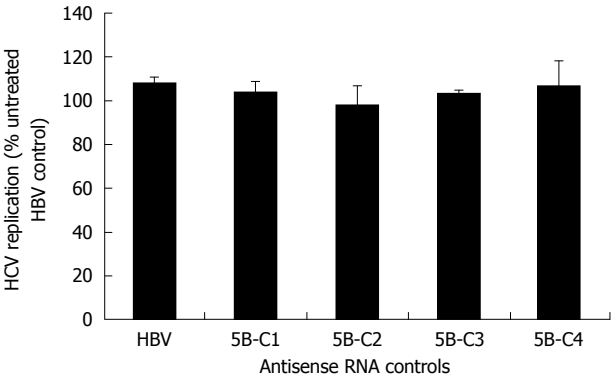


Figure 7 HCV replication after transfection of single-stranded RNA fragments corresponding to specific regions of the 5B-74 mimic in JFH-1-infected Huh7.5 cells. Cells stably infected with JFH-1 HCV were transfected separately with single-stranded fragments spanning the entire length of the 5B-74 mimic. HCV RNA levels were assayed by real-time RT-PCR. Assays were performed in triplicate and results expressed as mean ± SD of HCV replication as percent of unrelated (HBV) control.

conclusion that the 5B-74 and 5B-46 mimics inhibited HCV replication not by sequence complementarity, but by conformational attributes.

DISCUSSION

Previous studies have shown that overexpression of RNA analogs in infected cells can result in sequestration of essential factors. For example, suppression of human immunodeficiency virus-1 replication in cell models has been demonstrated by expression of structural mimics of the TAR element[40], RRE structure[41], and primer binding sequence[42]. HCV RNA has a number of CREs that could potentially be inhibited by structural RNA mimics. Structural mimics based on the HCV IRES were recently shown to inhibit HCV translation *in vitro* and in replicon models[10]. However, no effect on RNA replication was observed in the latter, possibly because of the bicistronic nature of the replicon.

It has been shown previously that double-stranded RNA can induce synthesis of cytokines including interferons which have antiviral effects. However, stimulation of interferon production has been reported to be dependent on the length of the RNA, with duplexes greater than 30 bp having been found to be most efficient[43]. Many studies have shown that smaller siRNAs of 20-21 base pairs (bp) have purely RNAi activity. In the current study, for the 5B-74, 5B-46, and 5B-100 mimics, the longest double-stranded regions were only 9 bp, followed by 8, 6 and 3 bp. Furthermore, the EC mimic was also predicted to have 9, 8, 6 and 3 bp regions. Yet, EC had essentially no significant inhibitory activity. Therefore, while double-stranded RNA stimulation of interferon production cannot be entirely excluded, if it occurs, it is not likely to account for the observed inhibitory effects of the 5B mimics.

Recently, cell culture systems for *in vitro* replication and infectious virus production have been established based on full-length HCV genotype 2a RNA[36,38,44,45]. This infectious HCV possesses 21% differences compared to the HCV 1b subgenomic replicon, BB7. Nucleotide sequences involved in the kissing-loop interaction are conserved between JFH-1 and BB7. However, mutations in other regions may affect this interaction by disrupting the RNA secondary structure[46]. It is interesting to note that small RNAs corresponding to 2 domains of HCV 2a, which differed from those in HCV 1b in 13 of 44 nucleotides and 6 of 48 nucleotides, respectively, inhibited the replication of replicon RNA from pLMH14 (HCV 1b) by 48.3% and 37.1%, respectively indicating that HCV 2a-derived RNAs could cross-interact with HCV 1b components despite the sequence differences between the elements of the 2 viruses[47]. Based on this finding, and the fact that most of the nucleotide substitutions in one domain from HCV 2a were compensatory bp changes, Zhang *et al*[47] hypothesized that the structure rather than the primary sequence was important for its function.

Viral replication has been shown to require the interaction of host factors with viral RNA and/or proteins. For HCV, several RNA-binding proteins, including L autoantigen[48], NS1-associated protein 1[49], polypyrimidine tract binding protein[50] and cyclophilin B[22] have been shown to bind to HCV RNA. Therefore, while we have focused on effects of mimics on HCV replication, because other vital HCV activities also depend on protein-RNA interactions, it is possible that the strategy for inhibition of replication by structural mimicry could be extended to other targets.

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COMMENTS

Background

Chronic hepatitis C virus (HCV) infection is a leading cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. Current therapeutic options are limited and associated with significant adverse effects. Accordingly, there is a strong impetus to develop novel therapeutic options. Because HCV replicates through the interaction of its RNA-dependent RNA polymerase and its RNA template to generate progeny RNA, physical contact between the enzyme and genomic RNA is required. The authors hypothesized that mimics could be created with sufficient resemblance to the natural genomic structure to compete with the HCV genome, resulting in inhibition of replication.

Research frontiers

Previous studies have shown that overexpression of RNA analogs in infected cells can result in sequestration of essential factors. For example, suppression of human immunodeficiency virus-1 replication in cell models has been demonstrated by expression of structural mimics of the TAR element, RRE structure, and primer binding sequence. HCV RNA has a number of cis-acting replication elements that could potentially be inhibited by structural RNA mimics. Structural mimics based on the HCV internal ribosome entry site were recently shown to inhibit HCV translation *in vitro* and in replicon models.

Innovations and breakthroughs

The novel feature of this research is the discovery that stretches of RNA can

be made that have little sequence similarity to HCV. However, because of the retention of the predicted structure, they have been found to be highly potent and specific agents for inhibition of HCV replication.

Applications

Specific sequences inhibiting HCV replication without substantial HCV sequence identity may be a novel therapeutic option to circumvent the appearance of resistance arising from mutation of the viral genome.

Terminology

Single-stranded RNA structural mimics are a class of polynucleotides that can inhibit replication of the HCV and are designed to mimic the shape of natural viral nucleic acids, without requiring the matching of nucleic acid sequences.

Peer review

The study of Smolic *et al* described the antiviral effect of HCV structural mimics in replicon and infectious models. It is a very straightforward and concise paper. The results provided clear evidence of the inhibition of viral replication by these mimics, likely *via* conformational attributes. However, several issues regarding the mechanism of action should be addressed to further strengthen this study.

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ORIGINAL ARTICLE

Differentiation of focal liver lesions using three-dimensional ultrasonography: Retrospective and prospective studies

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Abstract

AIM: To differentiate focal liver lesions based on enhancement patterns using three-dimensional ultrasonography (3D US) with perflubutane-based contrast agent.

METHODS: Two hundred and eighty two patients with focal liver lesions, including 168 hepatocellular carcinomas (HCCs), 63 metastases, 40 hemangiomas and 11 focal nodular hyperplasias (FNHs), were examined by 3D US with perflubutane-based contrast agent. Tomographic ultrasound images and sonographic angiograms were reconstructed. Among 282 lesions, enhancement patterns of 163 lesions between January 2007 and October 2007 were analyzed retrospectively. Then from November 2007 to May 2008, compared with contrast-enhanced (CE) 2D US, CE 3D US was performed on 119 lesions for prospective differential diagnosis. Sensitivity, specificity, area under receiver operating characteristic curve (A_z) and inter-reader agreement were assessed.

RESULTS: With the tridimensional view, dominant enhancement patterns were revealed as diffuse enhancement or peripheral ring-like enhancement, followed with washout change for HCCs or metastases, respectively, and peripheral nodular enhancement or diffuse enhancement with spoke-wheel arteries, followed by persistent enhancement for hemangiomas or FNHs, respectively. At CE 3D US, the prospective differentiation of lesions showed sensitivity 92% (mean for two readers), specificity 91% and A_z value 0.95 for HCCs, 84%, 97%, and 0.95 for metastases, 91%, 98%, and 0.98 for hemangiomas and 80%, 99%, and 0.99 for FNHs, respectively, while good to excellent inter-reader agreement was achieved. No significant difference exists between prospective diagnosis accuracy at CE 3D US and that at CE 2D US.

CONCLUSION: CE 3D US provides a spatial perspective for liver tumor enhancement, and could help in differentiating focal liver lesions.

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Key words: Three-dimensional; Ultrasonography; Contrast agent; Liver; Neoplasms

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INTRODUCTION

Focal liver lesions are increasingly being discovered with the widespread use of diagnostic imaging modalities, and differentiation of various liver lesions is considered to be critical for determining treatment options. Tremendous advancements in imaging techniques, including ultrasonography (US), computed tomography (CT), and magnetic resonance imaging (MRI) have resulted in these modalities being accepted as effective and thus widely used to characterize focal liver lesions[1-5]. This reduces the necessity of invasive histopathological examination.

With the advantages of non-invasiveness, no radiation and flexible operation, contrast-enhanced (CE) US has become increasingly important in the detection and characterization of focal liver tumors over the past several years[6-13]. Microbubbles of ultrasound contrast agent approximately the same size as red blood cells move into vessels but not through the vascular endothelium into the interstitium, which provides exact information about vascularity by reflecting specific signals, while some contrast agents present a postvascular hepato-specific phase probably due to reticuloendothelial system phagocytosis or the adherence of microbubbles to hepatic sinusoids[14-17]. Sonazoid (Daiichi Sankyo, Tokyo, Japan), a newly-developed second-generation ultrasound contrast agent, which consists of microbubbles (mean diameter 2-3 μm) of a perfluorobutane gas stabilized by a phospholipid monolayer shell, allows continuous real-time CE imaging for more than 10 min, and improves the reproducibility and durability of CE US examination[18-20].

Recently, the advent of CE three-dimensional (3D) US, facilitating demonstration of the desired regions with dynamic information in spatial volume, has led to new applications of CE US imaging[21-24]. Different from the 2D images, CE 3D US acquires the data in a volume of interest (VOI) by automatically scanning with a desired angle and allows reconstruction of tomographic images in three orthogonal planes and rendered angiogram-like images. With the feature of illustration in three dimensions, CE 3D US has been proven to offer accurate and detailed visualization of the vascular characteristics of various focal liver tumors[25,26].

To our knowledge, although many studies on differentiation among various focal liver lesions have been conducted using CE 2D US[27-29] and recently a few using CE 2D US with Sonazoid[30], the exact value of CE 3D US with Sonazoid in the differential diagnosis of various focal liver lesions has not yet been clarified. Thus, in this study, in order to examine the potential role of CE 3D US in characterizing focal liver lesions, we retrospectively evaluated the enhancement patterns and the diagnostic criteria established using dominant enhancement patterns were then applied to differentiation among focal liver lesions in a prospective study.

MATERIALS AND METHODS

Patients

Institutional Review Board approval was obtained for this retrospective and prospective study and informed consent was obtained from all patients. The reference standards for focal liver lesions in this study were described as follows: hepatocellular carcinomas (HCCs) with diameters exceeding 2 cm were confirmed by typical findings on both dynamic multi-detector CT and CE MRI[31]; HCCs exceeding 2 cm in diameter without typical radiological findings and those less than 2 cm in diameter, as well as all the liver metastases, were confirmed using histopathological results from percutaneous biopsy or surgery; benign tumors were confirmed by typical appearance on either dynamic multi-detector CT or CE MRI with at least 6-mo follow-up by dynamic multi-detector CT or CE MRI, and those without typical findings were confirmed histopathologically.

Between January 2007 and October 2007, 183 consecutive patients known or suspected to have focal liver lesions were examined using CE 3D US. Among these, 163 patients (90 men and 73 women) with 163 focal liver tumors were retrospectively identified, based on the following enrollment criteria: satisfactory CE 3D US images were acquired without artifacts from the cardiac and respiratory motion, shadows of costal bones, and abdominal gas; the definitive diagnosis of lesions was confirmed according to reference standards; the lesions had not been treated previously. Twenty patients were excluded: 6 with HCCs and 6 with hepatic metastasis lesions lacking a histological diagnosis; 3 HCCs and 3 hemangiomas with serious artifacts on the images; 2 benign lesions suspected on clinical information and imaging follow-up, without typical radiological findings and histological results. For 58 patients with multiple lesions, only the largest one with CE 3D US images and confirmed diagnosis was selected for evaluation. These 163 lesions were reviewed in the retrospective study to summarize the dominant enhancement patterns, which would be the diagnostic criteria in the prospective study.

From November 2007 to May 2008, the prospective study was conducted using CE 3D US on 124 consecutive patients with suspicious focal liver lesions detected by prior conventional US or CT and after that, CE 2D US was performed on each patient. For 40 patients with multiple lesions, CE 3D US examination was performed on the largest one, and if the largest lesion was not suitable for scanning due to its location, the next largest lesion was selected. CE 2D US was performed on the same lesion scanned by CE 3D US. In the prospective study, the exclusion criteria were: patients unable to hold breath; lesions with inappropriate locations for acquiring CE 3D

Table 1 Clinical characteristics of patients and focal liver lesions in retrospective and prospective studies (mean ± SD)

	Retrospective study (n = 163)				Prospective study (n = 119)			
	HCC	Metastasis	Hemangioma	FNH	HCC	Metastasis	Hemangioma	FNH
No. of patients	98	35	24	6	70	28	16	5
No. of lesions	98	35	24	6	70	28	16	5
Age of patients (yr)	69.8 ± 6.5	64.8 ± 13.2	58.9 ± 11.2	54.2 ± 16.7	72.5 ± 7.3	69.2 ± 8.3	55.4 ± 14.1	52.8 ± 18.4
Liver cirrhosis								
Child-Pugh class A	78	0	1	0	52	0	0	0
Child-Pugh class B	20	0	0	0	18	0	0	0
Lesion diameter (mm)	30.1 ± 24.3	28.9 ± 15.5	31.5 ± 18.0	38.4 ± 12.0	29.1 ± 20.4	30.7 ± 21.1	33.7 ± 16.7	37.4 ± 15.0
Final diagnosis								
Surgery	11	15	2	0	6	12	0	0
Biopsy	29	20	0	1	19	16	0	1
Radiological imaging	58	0	22	5	45	0	16	4

HCC: Hepatocellular carcinoma; FNH: Focal nodular hyperplasia.

US images due to artifacts from costal bone shadows, heart motion or abdominal gas; patients with lesions requiring histopathological diagnosis according to a reference standard but in whom surgery or biopsy was not possible due to poor liver function or lack of consent. Finally, 119 patients (67 men and 52 women) with 119 liver lesions were enrolled, while 2 patients with HCCs less than 2 cm in diameter and one with metastases were excluded for lack of consent on biopsy, and one patient with HCC and one with hemangioma were excluded for the obstruction of costal bone shadow.

Final diagnosis and clinical characteristics are shown in Table 1. The metastases in the retrospective study included 17 from colon carcinomas, 6 from pancreatic carcinomas, 5 from intrahepatic cholangiocellular carcinomas (ICC), 4 from rectal carcinomas, 1 from an ileum carcinoma, 1 from a gallbladder carcinoma and 1 from an ovarian carcinoma. Metastases in the prospective study comprised 15 from colon carcinomas, 4 from pancreatic carcinomas, 4 ICCs, 2 from rectal carcinomas, 1 from an ileum carcinoma, 1 from an ovarian carcinoma, and 1 from a gastric carcinoma. In this study, we classified the ICCs as hepatic metastasis because differentiation of ICCs from metastasis is difficult using only radiological imaging enhancement patterns.

Data acquisition

In the retrospective study, using the LOGIQ 7 ultrasound imaging system and 4D3CL volume probe (GE Healthcare, Milwaukee, WI) with a 2.0-5.5 MHz frequency, CE 3D US was performed by one sonographer with 10 years experience in using abdominal CE US. In the prospective study, CE 3D US was performed by the same sonographer in the retrospective study. After 1 or 2 d using the LOGIQ 7 ultrasound imaging system and 3.5 CS convex probe (GE Healthcare, Milwaukee, WI), CE 2D US was performed on each lesion by the same sonographer at CE 3D US. In this study, the acquisition of 3D images was performed with a convex volume 4D3C-L probe which scans automatically with internal sectorial mechanical tilt movement to obtain the data. The scanning angle could be selected from 15° to 84°. In this study we used 30-70° depending on different tumor sizes and the duration for one scanning process varied from 4.5 to 21.3 s (mean time, 12 s).

Prior to both CE 2D US and CE 3D US scanning, all patients received an intravenous bolus injection of 0.2 mL Sonazoid followed by a 2 mL 5% glucose solution and subsequent infusion of 5% glucose solution at 10 mL/min. The coded harmonic angio (CHA) mode (mechanical index = 0.5-0.9) at 8 to 13 frames per second was used for both CE 2D US and CE 3D US scanning. The contrast sonography process included three phases, that is, an early phase (10-40 s after contrast medium injection), a middle phase (80-120 s after contrast medium injection) and a late phase (more than 5 min after contrast medium injection). During each phase, the CE US scanning was performed when the characteristic enhancement of focal liver lesions appeared.

Static 3D and Autosweep 3D functionalities, with which the LOGIQ 7 ultrasound imaging system is equipped, were used for image acquisition. Employing the different engineering features of these two functionalities, in the early phase, both Static 3D and Autosweep 3D were used to acquire 3D data, while in the two other phases only the Autosweep 3D functionality was used. A VOI with an adjustable scanning angle and size was determined before scanning to contain the desired region. The data acquired were stored as cineloops in the hard disk of the ultrasound imaging system.

Image reconstruction

3D image reconstruction was performed using the functionalities with which the LOGIQ 7 ultrasound system was equipped. In the VOI which was presented in an isotropic rectangular coordinate frame, the three orthogonal planes were referred to as plane A which could migrate from front to rear through the VOI, plane B from left to right and plane C from up to down. Tomographic ultrasound images (TUI) with presentation of several parallel slices in three orthogonal planes were reconstructed in three phases, while the number of slices that could be selected was 2, 4, or 6 for images obtained using Autosweep 3D, and 2, 4, or 9 for Static 3D. In

order to show the desired images, the range of distance between two slices in TUI could be adjusted if required. The mean times of these procedures were about 20 s. Sonographic angiogram images were reconstructed in an angio-like view during the early phase by using various rendering modes. The maximum intensity mode displaying the maximum grey values in the VOI, mixed with surface mode displaying the grey value on the surface of objects, was used to visualize tumor vessels and early tumor enhancement, while the average intensity mode displaying the average grey values in the VOI, mixed with surface mode, was employed to describe the tumor based on the predominantly unenhanced areas. The mean times of these procedures were about 45 s. TUI in three dimensions and sonographic angiogram images were stored with volume data in the hard disk of the ultrasound imaging system. In both the retrospective study and the prospective study, the reconstruction of all the images was completed by one operator with 10 years of experience in abdominal US and 1 year in CE 3D US, blinded to the final diagnosis and other related information on patients.

Image evaluation

Before the 3D image evaluations in both the retrospective and prospective studies, all readers were given training in the assessment of various enhancement patterns. Image evaluation was divided into two sections: in the first section, TUI in early, middle and late phases and sonographic angiograms in the early phase were reviewed separately in random order for all the lesions and the enhancement patterns in each phase were classified; in the second section, all images in three phases for each lesion were read consecutively to observe enhancement changes over time. In the prospective study, 2D images were read 2 wk after the 3D image review. All the images were reviewed on the ultrasound imaging system. The zoom function and arbitrary rotation of images are available in both TUI and sonographic angiograms.

In the retrospective study, images were reviewed independently by two readers (Numata K and Morimoto M) with 10 years of experience in liver US and 1.5 years of experience in CE 3D US imaging, who were blinded to final diagnosis and clinical and other radiological information. The readers were asked to classify the enhancement patterns in three phases and enhancement changes over time. In the early phase, the enhancement patterns consisted of tumor enhancement and tumor vessels: four tumor enhancement patterns including diffuse enhancement defined as homogeneous or heterogeneous tumor enhancement, peripheral ring-like enhancement defined as enhancement in the tumor periphery with a ring shape, peripheral nodular enhancement defined as enhancement in the tumor periphery with a nodular shape, and absence of tumor enhancement; four tumor vessel patterns including intratumoral vessels in the central portion of the tumor, peritumoral vessels at the periphery of the tumor, spoke-wheel arteries defined as a centrally located artery with centrifugal stellate branching, and absence of vessels. In the middle phase, four patterns were classified based on tumor enhancement: diffuse enhancement, peripheral ring-like enhancement, peripheral nodular enhancement and perfusion defect. In the late phase, the hypoechoic pattern was defined as all or part of the lesion having low echogenicity as compared to the surrounding liver parenchyma, and the isoechoic pattern as the entire lesion having echogenicity equal to that of the surrounding liver parenchyma. The enhancement changes over time were assessed: washout, early enhancement greater than that of the liver parenchyma and enhancement in the middle or late phase less than that of the liver parenchyma; persistence, whatever the enhancement pattern in the early phase was, enhancement of the lesion in the middle and late phases was at least equal to that of the liver parenchyma; absence of washout and persistence, no distinct enhancement was detected in any of the three phases.

Evaluations of 153 lesions by two readers were in concordance. For 10 lesions, enhancement patterns evaluated by two readers were different but a consensus was finally reached. After the pattern classification, the combination of enhancement patterns in the three phases was summarized and the positive predictive value (PPV) was calculated. The combined enhancement patterns with PPV more than 50% served as diagnostic criteria for the analysis in the prospective study.

In the prospective study, two readers (Nozaki A and Luo W) with 8 and 5 years of experience in CE US of the liver and 1 year of experience in CE 3D US imaging, blinded to the final diagnosis and clinical and other radiological information, read CE 3D US images and differentiated focal liver lesions according to the diagnostic criteria established in the retrospective study. After reading 2D images, these two readers also made a diagnosis of each lesion according to the criteria previously established (Table 2)[27,28,32,33].

On 3D images and 2D images, for each diagnosis, i.e. HCC, metastasis, hemangioma, and focal nodular hyperplasia (FNH), a four-point scale was used to grade diagnostic confidence. For instance, in HCCs, grade 1 was defined as "probably not a HCC lesion", grade 2 as "possibly not a HCC lesion", grade 3 as "possibly a HCC lesion" and grade 4 as "probably a HCC lesion". The confidence level of each reader was compared in consideration of the final diagnosis by two physicians (Kondo M and Tanaka K). True-positive (TP) cases were considered to be those assigned as grade 3 to 4 and verified by reference standards as actually being positive diagnoses. False-positive (FP) cases were considered to be those assigned as grade 3 to 4 but verified as actually being negative diagnoses. True-negative (TN) cases were considered to be those assigned as grade 1 to 2 by the reader, and verified as actually being negative diagnoses. False-negative (FN) cases were considered to be those assigned as grade 1 to 2 but verified as actually being positive diagnoses.

Table 2 Diagnostic criteria for focal liver lesions depicted by CE 2D US

Lesion	Enhancement patterns		
	Early phase	Middle phase	Late phase
HCC	Intratumoral vessels with early homogeneous or heterogeneous enhancement, or intratumoral vessels alone	Homogeneous or heterogeneous enhancement	Hypoechoic lesion
Metastasis	Peritumoral vessels with early peripheral ring like enhancement	Peripheral ring like enhancement or perfusion defect	Hypoechoic lesion
Hemangioma	Peripheral nodular enhancement	Peripheral nodular enhancement, with centripetal progression	Isoechoic lesion, with centripetal progression
FNH	Spoke-wheel arteries with early homogeneous enhancement	Homogeneous enhancement	Isoechoic lesion with central scar

CE: Contrast-enhanced; 2D US: Two-dimensional ultrasonography.

Statistical analysis

The data analysis was performed by using SPSS software (version 11.0; SPSS, Tokyo, Japan). In the retrospective study, SE was defined as the probability of the given enhancement pattern appearing in a reference disease, while SP indicated the probability of the absence of a given enhancement pattern for negative diagnoses. Prior probability was calculated by dividing the numbers of HCCs, metastases, hemangiomas and FNHs by the total number of tumors (163 tumors). According to Bayes theorem, the PPV of the combination of enhancement patterns for each tumor category was calculated based on sensitivity (SE), specificity (SP), and prior probability (PP) using the formula: PPV = SE × PP/[SE × PP + (1 - SP)× (1 - PP)].

In the prospective study, SE was calculated as TP/(TP + FN) and SP was calculated as TN/(FP + TN). The ROC curve was fitted to each reader's confidence level by using a maximum likelihood estimation program (ROCKIT 0.9B; http://www-radiology.uchicago.edu/krl/KRL_ROC). Area under the ROC curve (A_z) with the 95% confidential interval was calculated for each modality to measure the overall diagnostic performance. κ values were used to assess inter-reader agreement in characterizing focal liver tumors. Agreement was graded as poor (< 0.20), moderate (0.20 to 0.40), fair (0.40 to 0.60), good (0.60 to 0.80), or excellent (0.80 to 1.00).

RESULTS

Retrospective study

The enhancement patterns of 163 focal liver lesions are summarized in Table 3. In the early phase, 90% (88 of 98) of HCCs showed diffuse enhancement with intratumoral vessels (Figure 1A and B), 60% (21 of 35) of liver metastases peripheral ring-like enhancement with intratumoral or peritumoral vessels (Figure 2A), 79% (19 of 24) of hemangiomas peripheral nodular enhancement with peritumoral vessels or absence of vessels (Figure 3A and B), and 100% (6 of 6) of FNHs diffuse enhancement with spoke-wheel arteries (Figure 4A-C). In the middle phase, 93% (91 of 98) of HCCs showed diffuse enhancement (Figure 1C) and 69% (24 of 35) of metastases peripheral ring-like enhancement (Figure 2B), 75% (18 of 24) of hemangiomas peripheral nodular enhancement (Figure 3C) and 25% (6 of 24) diffuse enhancement, and 100% (6 of 6) of FNHs diffuse enhancement. In the late phase, 137 of 163 lesions were hypoechoic (Figures 1D, 2C, 2D and 3D), while the others were isoechoic (Figure 4D). Washout of enhancement was detected mainly in 121 of 133 malignant lesions (Figures 1 and 2), while enhancement persistence was detected mainly in 29 of 30 benign lesions (Figures 3 and 4). One HCC and two metastasis lesions were not enhanced from the early to the late phase and were assessed as showing absence of washout and persistence. The combination of enhancement patterns in three phases with PPV above 50% were summarized for each tumor category in Table 4 as the dominant enhancement patterns for establishing diagnostic criteria on 3D images in prospective study.

Prospective study

For CE 3D US, the diagnostic criteria for HCCs resulted in a SE of 93% (mean for two readers), SP of 91% and A_z value of 0.95, while those for metastases showed a SE of 84%, SP of 97% and A_z value of 0.95 in the prospective study. The combined enhancement patterns for hemangiomas as diagnostic criteria resulted in a SE of 91%, SP of 98% and A_z value of 0.98, while the dominant enhancement patterns for FNHs as diagnostic criteria resulted in a SE of 80%, SP of 99%, and A_z value of 0.99. Good to excellent inter-reader agreement was thus achieved for HCCs (κ = 0.86), metastases (κ = 0.83), hemangiomas (κ = 0.79) and FNHs (κ = 0.76).

For CE 2D US, sensitivity, specificity and A_z value of differential diagnosis were shown in Table 5. There were no significant differences between sensitivity, specificity and A_z value on CE 2D US and those on CE 3D US ($P > 0.05$). For HCCs, there was an SE of 92%, SP of 87% and A_z value of 0.95 (mean of two readers), while an SE of 84%, SP of 97% and A_z value of 0.94 were recorded for metastasis. For hemangioma, there was an SE of 84.5%, SP of 98% and A_z value of 0.95. For FNH, an SE of 70%, SP of 98% and A_z value of 0.98 were presented. SE, SP and A_z value of hemangioma and FNH on CE 2D US appeared lower than those on CE 3D US.

Table 3 Enhancement patterns using CE 3D US in three phases: positive predictive value in retrospective study

No.	Enhancement patterns					Positive predictive value			
	Early phase		Middle phase	Late phase	Enhancement changes	HCC	Metastasis	Hemangioma	FNH
	Tumor enhancement	Tumoral vessels	Tumor enhancement	Tumor echogenicity					
1	Diffuse	Intratumoral	Diffuse	Hypoechoic	Washout	0.98 (81)	0.01 (1)	0.01 (1)	0.00 (0)
2	Diffuse	Intratumoral	Diffuse	Isoechoic	Persistence	0.60 (3)	0.00 (0)	0.40 (2)	0.00 (0)
3	Diffuse	Intratumoral	Peripheral ring-like	Hypoechoic	Washout	0.33 (2)	0.67 (4)	0.00 (0)	0.00 (0)
4	Diffuse	Intratumoral	Perfusion defect	Hypoechoic	Washout	0.67 (2)	0.33 (1)	0.00 (0)	0.00 (0)
5	Diffuse	Spoke-wheel arteries	Diffuse	Isoechoic	Persistence	0.00 (0)	0.00 (0)	0.00 (0)	1.00 (6)
6	Peripheral ring-like enhancement	Intratumoral	Diffuse	Isoechoic	Persistence	1.00 (3)	0.00 (0)	0.00 (0)	0.00 (0)
7	Peripheral ring-like enhancement	Intratumoral	Peripheral ring-like	Hypoechoic	Washout	0.14 (1)	0.86 (6)	0.00 (0)	0.00 (0)
8	Peripheral ring-like enhancement	Peritumoral	Peripheral ring-like	Hypoechoic	Washout	0.00 (0)	1.00 (12)	0.00 (0)	0.00 (0)
9	Peripheral ring-like enhancement	Peritumoral	Perfusion defect	Hypoechoic	Washout	0.00 (0)	1.00 (3)	0.00 (0)	0.00 (0)
10	Peripheral nodular	Peritumoral	Diffuse	Isoechoic	Persistence	0.00 (0)	0.00 (0)	1.00 (1)	0.00 (0)
11	Peripheral nodular	Peritumoral	Peripheral nodular	Hypoechoic	Persistence	0.00 (0)	0.00 (0)	0.67 (2)	0.00 (0)
12	Peripheral nodular	Peritumoral	Peripheral nodular	Isoechoic	Persistence	0.00 (0)	0.00 (0)	1.00 (1)	0.00 (0)
13	Peripheral nodular	Peritumoral	Peripheral nodular	Hypoechoic	Washout	0.00 (0)	0.33 (1)	0.00 (0)	0.00 (0)
14	Peripheral nodular	Absence	Diffuse	Isoechoic	Persistence	0.00 (0)	0.00 (0)	1.00 (2)	0.00 (0)
15	Peripheral nodular	Absence	Peripheral nodular	Hypoechoic	Persistence	0.00 (0)	0.00 (0)	1.00 (8)	0.00 (0)
16	Peripheral nodular	Absence	Peripheral nodular	Isoechoic	Persistence	0.00 (0)	0.00 (0)	1.00 (5)	0.00 (0)
17	Absence	Intratumoral	Diffuse	Isoechoic	Persistence	1.00 (3)	0.00 (0)	0.00 (0)	0.00 (0)
18	Absence	Intratumoral	Diffuse	Hypoechoic	Washout	1.00 (1)	0.00 (0)	0.00 (0)	0.00 (0)
19	Absence	Intratumoral	Peripheral ring-like	Hypoechoic	Washout	0.00 (0)	1.00 (1)	0.00 (0)	0.00 (0)
20	Absence	Intratumoral	Perfusion defect	Hypoechoic	Washout	1.00 (1)	0.00 (0)	0.00 (0)	0.00 (0)
21	Absence	Peritumoral	Peripheral ring-like	Hypoechoic	Washout	0.00 (0)	1 .00(1)	0.00 (0)	0.00 (0)
22	Absence	Peritumoral	Perfusion defect	Hypoechoic	Washout	0.00 (0)	1.00 (3)	0.00 (0)	0.00 (0)
23	Absence	Absence	Perfusion defect	Hypoechoic	Absence	0.33 (1)	0.67 (2)	0.00 (0)	0.00 (0)
24	Absence	Absence	Peripheral nodular	Hypoechoic	Persistence	0.00 (0)	0.00 (0)	1.00 (2)	0.00 (0)

Positive predictive values are presented with number of lesions in parentheses.

Table 4 Sensitivity, specificity and A_z value for differential diagnosis based on CE 3D US in prospective study

Focal liver tumors	Diagnostic criteria based on combined enhancement patterns		Sensitivity	Specificity	A_z value
HCC	1, 2, 4, 6, 17, 18, 20	Reader 1	91 (64/70)	90 (44/49)	0.94 (0.88-0.95)
		Reader 2	94 (66/70)	92 (45/49)	0.96 (0.91-0.98)
Metastasis	3, 7, 8, 9, 13, 19, 21, 22, 23	Reader 1	86 (24/28)	97 (88/91)	0.95 (0.89-0.98)
		Reader 2	82 (23/28)	96 (87/91)	0.94 (0.89-0.97)
Hemangioma	10, 11, 12, 14, 15, 16, 24	Reader 1	94 (15/16)	98 (101/103)	0.98 (0.94-1.00)
		Reader 2	88 (14/16)	97 (100/103)	0.97 (0.90-1.00)
FNH	5	Reader 1	80 (4/5)	98 (112/114)	0.99 (0.95-1.00)
		Reader 2	80 (4/5)	99 (113/114)	0.98 (0.87-1.00)

Combined enhancement patterns for diagnostic criteria are summarized according to the serial numbers in Table 3. Sensitivity and specificity data are presented as percentages with numbers of tumors in parentheses, and A_z values are presented with 95% confidence intervals in parentheses.

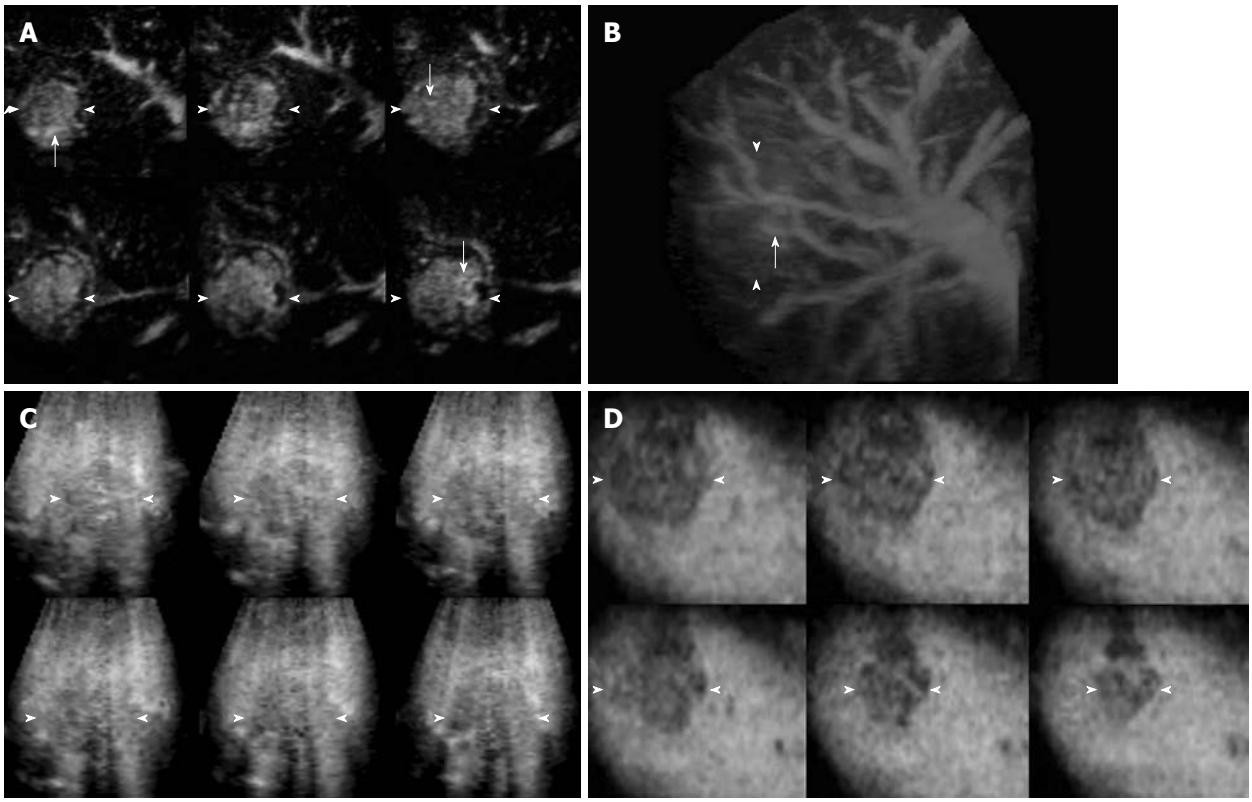


Figure 1 Contrast-enhanced (CE) three-dimensional ultrasonography (3D US) images of the liver in a 57-year-old man with hepatocellular carcinoma (HCC) in the anterior superior segment of the right lobe. A, B: Tomographic ultrasound image with slice distance 1.5 mm in plane A (A) and the sonographic angiogram rendered by maximum intensity with surface mode (B) show diffuse enhancement with intratumoral vessels (arrows) in the early phase; C: Tomographic ultrasound image with slice distance 2.0 mm in plane B shows diffuse enhancement in the middle phase; D: Tomographic ultrasound image with slice distance 2.0 mm in plane C shows hypoechoic pattern in the late phase. Enhancement change of washout was detected in this lesion. Arrowheads indicate the tumor margin.

Table 5 Sensitivity, specificity and A_z value for differential diagnosis based on CE 2D US in prospective study

Lesion	Sensitivity	Specificity	A_z value (95% CI)
HCC			
Reader 1	89 (62/70)	88 (43/49)	0.95 (0.89-0.98)
Reader 2	94 (66/70)	86 (42/49)	0.95 (0.90-0.98)
Metastasis			
Reader 1	86 (24/28)	96 (87/91)	0.93 (0.85-0.97)
Reader 2	82 (23/28)	98 (89/91)	0.95 (0.88-0.98)
Hemangioma			
Reader 1	81 (13/16)	97 (100/103)	0.94 (0.80-0.99)
Reader 2	88 (14/16)	99 (102/103)	0.95 (0.73-1.00)
FNH			
Reader 1	60 (3/5)	97 (111/114)	0.97 (0.74-1.00)
Reader 2	80 (4/5)	98 (112/114)	0.98 (0.66-1.00)

Sensitivity and specificity data are presented as percentages with numbers of tumors in parentheses.

DISCUSSION

Our study explored the potential role of CE 3D US, a newly-developed imaging modality, in presenting various tumor enhancement patterns and further characterizing different types of focal liver lesions. In this study, we used Sonazoid with CHA mode and high mechanical index contrast conditions, which allowed sensitive observation of vessels in the early phase by eliminating microbubbles in microvessels but not those in relatively large vessels, such as tumor vessels and portal veins[19].

In a previous study, we explored the visualization methods of CE 3D US for demonstrating focal liver lesions[24]. Moreover, in a retrospective study, according to the diagnostic criteria based on our experience and that described in the literatures, we assessed the diagnostic value of CE 3D US for characterization of focal liver lesions, in comparison with that of CE 3D CT[22]. Further, in the present study, we performed a retrospective study to clarify the enhanced patterns of focal liver lesions on CE 3D US, and the combined enhancement patterns with PPV more than 50% served as diagnostic criteria. According to these diagnostic criteria, we prospectively evaluated CE 3D US for characterizing liver lesions.

On CE 3D US, sonographic angiograms rendered using maximum intensity mode could be used to delineate tumor enhancement and present the spatial distribution of tumor vessels. Spoke-wheel arteries were regarded as a typical characteristic of FNHs on CE imaging in previous studies[34-36]. CE 3D US facilitated categorization of spoke-wheel arteries and provided a tridimensional view of the stellate branches. With consensus of the two readers, all 6 FNHs in the retrospective study were detected based on spoke-wheel arteries followed by persistent enhancement. In the prospective study, except for one FNH without distinctly detectable of spoke-wheel arteries, 4 of the other 5

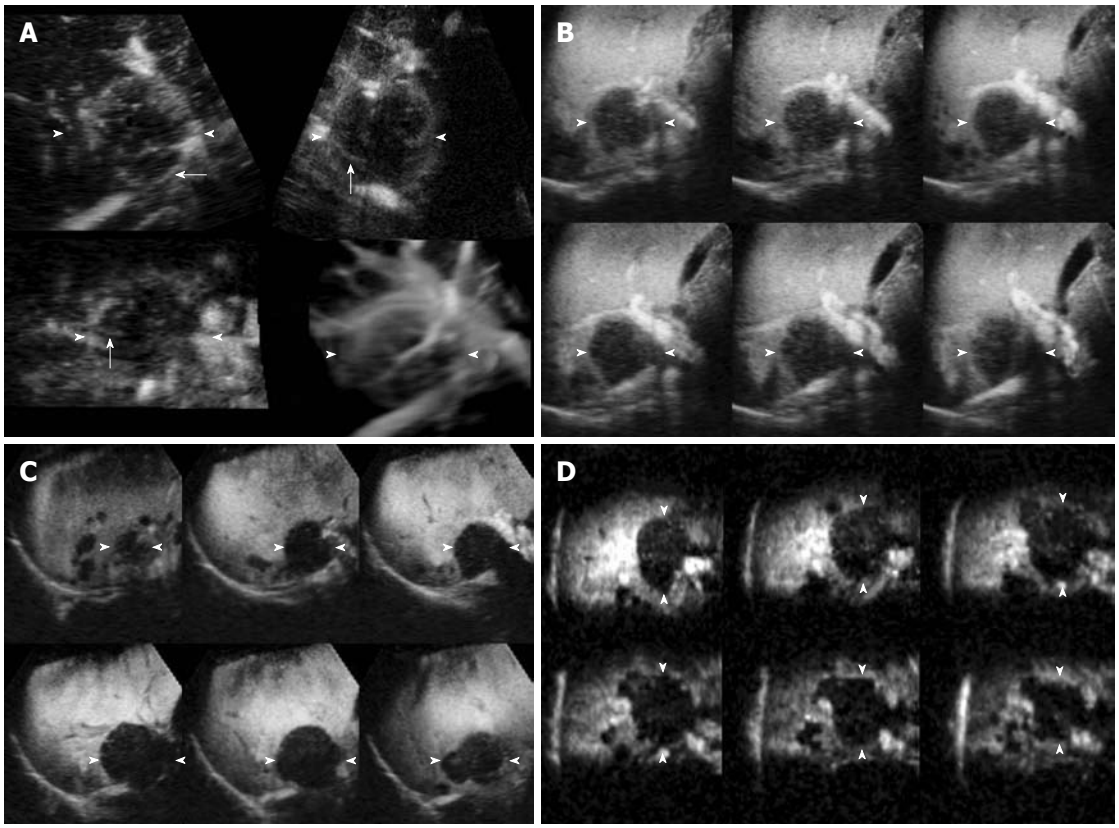


Figure 2 CE 3D US images of the liver in a 64-year-old woman with metastases from pancreatic carcinoma in the anterior superior segment of the right lobe. A: Images in the early phase show peripheral ring-like enhancement with peritumoral vessels (arrows) in plane A (upper left), plane B (upper right), plane C (lower left) and the sonographic angiogram rendered by average intensity with surface mode (lower right); B: Tomographic ultrasound image with slice distance 2.0 mm in plane A shows peripheral ring-like enhancement in the middle phase; C, D: Tomographic ultrasound images (TUI) in plane A with slice distance 2.5 mm (C) and that in plane C with slice distance 2.0 mm (D) show hypoechoic pattern in the late phase. Enhancement change of washout was detected in this lesion. Arrowheads indicate the tumor margin.

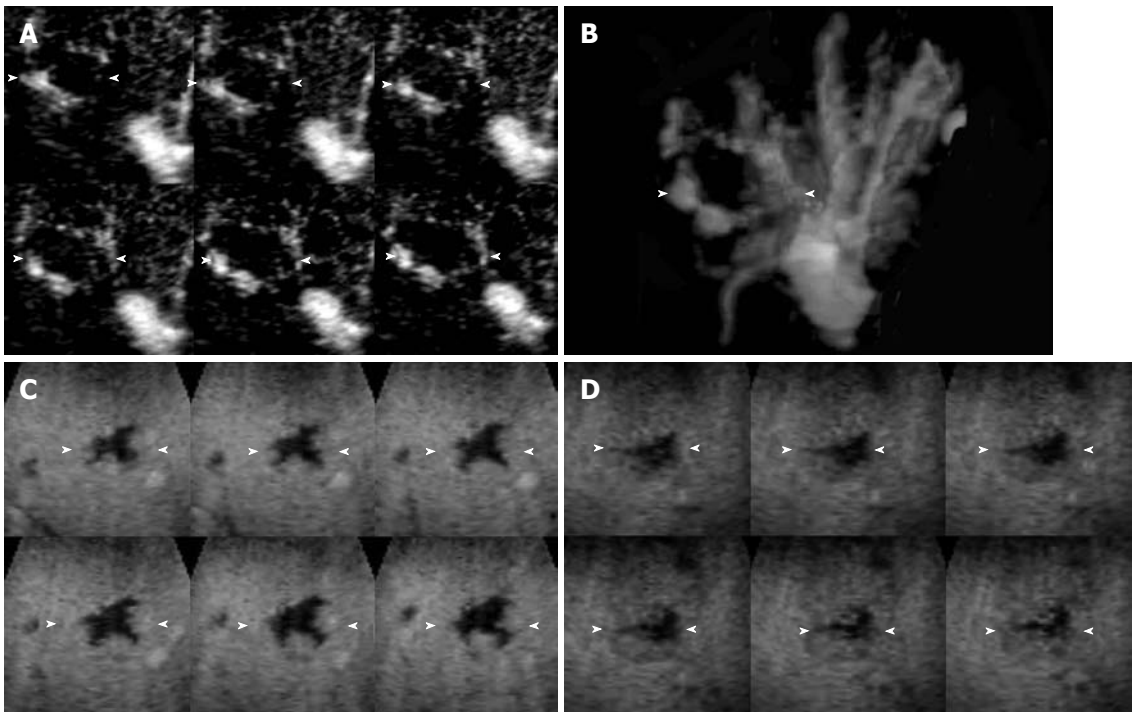


Figure 3 CE 3D US images of the liver in a 59-year-old woman with a hemangioma in the anterior inferior segment of the right lobe. A, B: Tomographic ultrasound image in plane A with slice distance 1.0 mm (A) and sonographic angiogram rendered by average intensity with surface mode (B) show peripheral nodular enhancement without distinct tumor vessels in the early phase; C: Tomographic ultrasound image in plane B with slice distance 1.0 mm shows peripheral nodular enhancement in the middle phase; D: Tomographic ultrasound image with slice distance 1.0 mm in plane B with slice distance 1.0 mm shows hypoechoic pattern in the late phase. Persistent enhancement over time was detected in this lesion. Arrowheads indicate the tumor margin.

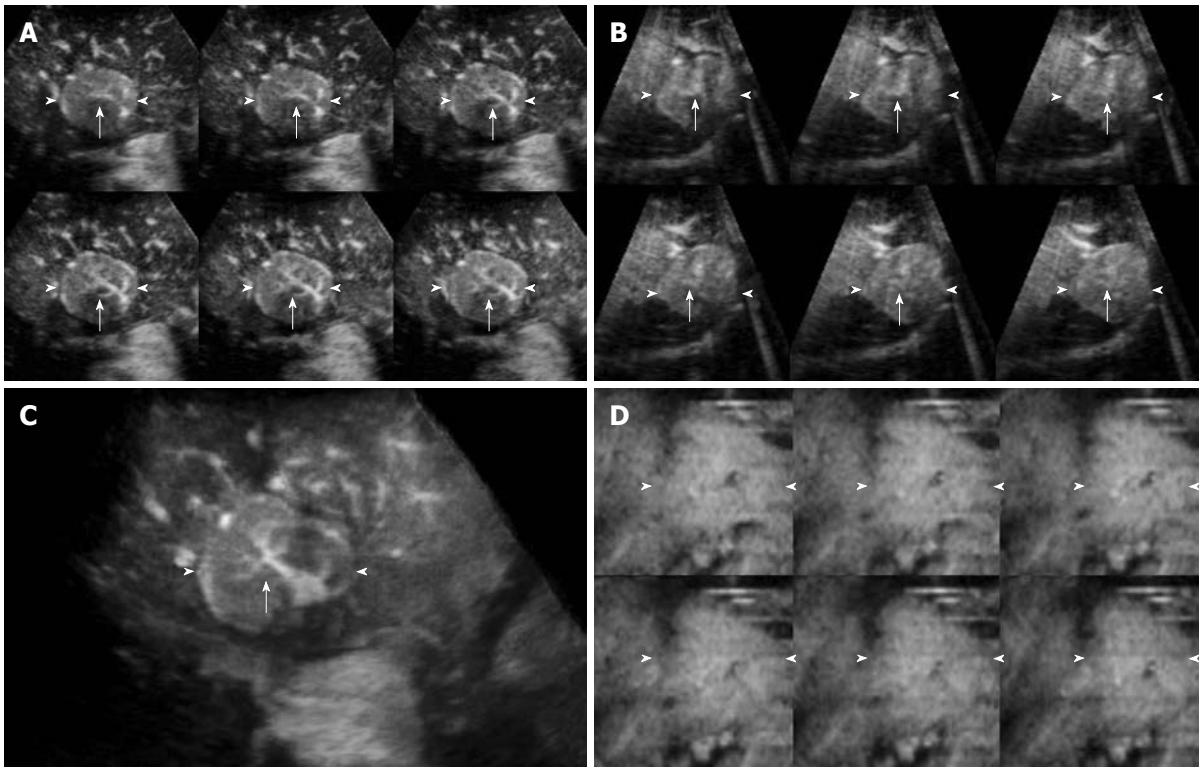


Figure 4 CE 3D US images of the liver in a 77-year-old man with FNH in the medial segment of the left lobe. A-C: TUI in plane A with slice distance 1.5 mm (A) and plane B with slice distance 1.5 mm (B), and the sonographic angiogram rendered by maximum intensity with surface mode (C) show diffuse enhancement with spoke-wheel arteries (arrows) in the early phase; D: TUI in plane C with slice distance 2.5 mm show isoechoic pattern in the late phase and hypoechoic regions within the tumor indicate central scars. Persistent enhancement over time was detected in this lesion. Arrowheads indicate the tumor margin.

lesions were diagnosed correctly. For the lesions with peripheral enhancement, sonographic angiograms rendered using average intensity modes delineated unenhanced portions in the lesions with hypoechoic appearance to make the tumor distinguishable from the surrounding liver parenchyma. Another feature of CE 3D US was the application of tomographic images presented in several parallel slices to multiplanar reconstruction, which allowed the reader to evaluate enhancement patterns in different portions of the lesion simultaneously. In this study, although there were no significant differences between the prospective diagnosis at CE 3D US and that at CE 2D US, CE 3D US created a spatial and easily understood view for both hemodynamic and morphologic evaluation of focal liver tumors, which were formed only in the doctors' imagination by 2D imaging using complex acquisition methods. The good to excellent inter-reader agreement in our previous study about CE 3D US as a means of presenting characteristic enhancement of HCCs has indicated CE 3D US can exhibit the characteristic enhancement of HCC tumors objectively[34].

In the present study, with analysis of combination of the enhancement in three phases at CE 3D US, the dominant patterns were used as the diagnostic criteria for individual category, and prospective differentiation yielded a good sensitivity, specificity, high A_z value, and good to excellent inter-reader agreement, which revealed the potential usage of CE 3D US in differentiating various focal liver lesions.

In the late phase in the retrospective study, 91% of malignant tumors (88 HCCs and 33 metastases) with washout changes appeared hypoechoic, while 53% of benign tumors (10 hemangiomas and 6 FNHs) with persistent enhancement appeared isoechoic. Neither isoechoic metastases nor hypoechoic FNHs were found in the late phase. There were 13 hemangiomas exhibiting hypoechoic findings in the late phase, and 12 of these 13 lesions were detected with incomplete fill-in and persistent enhancement, while one hemangioma was detected with early diffuse enhancement and washout change and was confirmed by the histological results on surgery. Vascularity in the early and middle phases appeared to be important for differentiation of focal liver lesions, and enhancement in the late phase with enhancement changes over time also facilitated characterizing the benign and malignant lesions[28,37].

This study has some limitations. First, CE 3D US on focal liver lesions has shortcomings including artifacts from the heart or respiratory motion, shadows of costal bones, and interference from abdominal gas, and so on. We minimized the influence of artifacts by requiring patients to hold their breath during the scanning procedure, selecting the location of the volume transducer, mediating the size and position of VOI, adjusting the scanning angle, and reconstructing the sonographic angiogram with appropriate rendering modes. Nevertheless, in some cases, artifacts on CE 3D US images might impact the readers' judgments and these lesions were thus excluded.

Second, in each case, only one lesion was evaluated and we did not assess the diagnostic capability and detectability of multiple lesions with CE 3D US. Third, the FNH cases in this study were few because FNHs have a relatively low prevalence and Sonazoid has been available in Japan for less than 2 years. More cases need to be examined in a future investigation.

In summary, our present results indicate CE 3D US with the contrast agent Sonazoid to offer a novel and useful means of presenting the vascularity characteristics three-dimensionally and differentiation of focal liver lesions with good diagnostic capability. These features of CE 3D US are anticipated to be of benefit in clinical diagnosis.

COMMENTS

Background

Recently, contrast-enhanced (CE) ultrasound (US) appeared as an important modality to show the vascularity in the areas of interest, and has been used widely in clinical diagnosis of liver lesions. Three dimensional ultrasonography (3D US) allows three orthogonal planes to spatially demonstrate the features of subjects, which has been frequently used in fetal US. Different from the 2D images, CE 3D US acquires the data in a volume of interest (VOI) by automatically scanning with a desired angle and allows reconstruction of tomographic images in three orthogonal planes and renders angiogram-like images. The combination of 3D US and CE US can present the enhancement of lesions in three dimensions and also in parallel slices by multiple- planar visualization.

Research frontiers

With the advantages of non-invasiveness, no radiation and flexible operation, CE US has become increasingly important in the detection and characterization of focal liver tumors over the past several years. Sonazoid (Daiichi Sankyo, Tokyo, Japan), a newly-developed second-generation US contrast agent, which consists of microbubbles (mean diameter 2-3 μm) of a perfluorobutane gas stabilized by a phospholipid monolayer shell, allows continuous real-time CE imaging for more than 10 min, and improves the reproducibility and durability of CE US examination.

Innovations and breakthroughs

To the authors' knowledge, although many studies on differentiation among various focal liver tumors have been conducted using CE 2D US and recently a few using CE 2D US with Sonazoid, the exact value of CE 3D US with Sonazoid in the differential diagnosis of various focal liver tumors has not yet been clarified. Thus, in this study, in order to examine the potential role of CE 3D US in characterizing focal liver tumors, the authors retrospectively evaluated tumor enhancement patterns, and the diagnostic criteria established using dominant enhancement patterns were then applied to differentiation among focal liver tumors in a prospective study.

Applications

The study's results suggest that CE 3D ultrasound provides a spatial perspective for liver tumor enhancement, and could help in differentiating focal liver tumors.

Terminology

Contrast enhanced ultrasound: the imaging modality of using US scanning while microbubbles serving as contrast agent are injected into vessels in order to improve the signals of red blood cells. Using this modality, the vascularity of VOI was depicted exactly and hemodynamics was demonstrated clearly.

Peer review

The aim of this study is to differentiate focal liver lesions based on enhancement patterns using 3D US with perflubutane-based contrast agent. The paper is written in a good English form, and is properly structured.

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ORIGINAL ARTICLE

Direct *in vivo* injection of ^{131}I-GMS and its distribution and excretion in rabbit

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Abstract

AIM: To explore the distribution and metabolism of ^{131}I-gelatin microspheres (^{131}I-GMSs) in rabbits after direct injection into rabbits' livers.

METHODS: Twenty-eight healthy New Zealand rabbits were divided into seven groups, with four rabbits per group. Each rabbit's hepatic lobes were directly injected with 41.336 ± 5.106 MBq ^{131}I-GMSs. Each day after ^{131}I-GMSs administration, 4 rabbits were randomly selected, and 250 μL of serum was collected for γ count. Hepatic and thyroid functions were tested on days 1, 4, 8, 16, 24, 32, 48 and 64 after ^{131}I-GMSs administration. Single-photon emission computed tomography (SPECT) was taken for each group on days 0, 1, 4, 8, 16, 24, 32, 48, 64 after ^{131}I-GMSs administration. A group of rabbits were sacrificed respectively on days 1, 4, 16, 24, 32, 48, 64 after ^{131}I-GMSs administration. Their livers were taken out for histological examination.

RESULTS: After ^{131}I-GMSs administration, the nuclide was collected in the hepatic area with microspheres. The radiation could be detected on day 48 after ^{131}I-GMSs administration, and radiography could be seen in thyroid areas in SPECT on days 4, 8, 16 and 24. One day after ^{131}I-GMSs administration, the liver function was damaged but recovered 4 d later. Eight days after ^{131}I-GMSs administration, the levels of free triiodothyronine and free thyroxin were reduced, which restored to normal levels on day 16. Histological examination showed that the microspheres were degraded to different degrees at 24, 32 and 48 d after ^{131}I-GMSs administration. The surrounding parts of injection points were in fibrous sheathing. No microspheres were detected in histological examination on day 64 after ^{131}I-GMSs administration.

CONCLUSION: Direct *in vivo* injection of ^{131}I-GMSs is safe in rabbits. It may be a promising method for treatment of malignant tumors.

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Key words: ^{131}I; Label; Gelatin microspheres; Animal; Rabbit; Hepatic; Direct injection

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INTRODUCTION

The treatment of malignant tumors in hepatic, biliary and pancreatic systems has been and will always be a tough and key part of general surgery[1-3]. Surgical removal has been considered a positive approach for most of malignant tumors in these systems[4]. But the success rate of surgical removal is very low and the prognosis after operation is unsatisfactory[5-8]. For example, the number of cases of primary hepatic carcinoma in China was around 384 119 in 2005[9-12] and the number of deaths was 357 624[13-15], only 10%-30% are related to surgical removal, and 25% of them survived over 5 years[16]. The number of cases of pancreatic cancer in America was around 42 470 and the number of death was around 35 240 in 2009[17]. Surgical procedure has much limitations for treatment of malignant cancers. Many patients with malignant tumors in the three systems at middle to advanced stages are in the urgent need of new and effective non-surgical treatment.

Radionuclide labeled microspheres and seeds are the new progress made in the area of tumor therapy and interventional radiotherapy. It has now become a fast developing sub-field of medicine combining nuclear medicine and oncotherapy. Nuclide microspheres under study over recent years include ^{90}Y glass and resin microsphere[18-20], ^{188}Re glass microsphere[21], ^{32}P glass microsphere[22,23] and ^{166}Ho glass microsphere[24]. The ^{90}Y glass microsphere is being increasingly recognized by the medical community as an important strategy for the treatment of primary and secondary neoplasm, which was officially approved in 1999 in America and Canada for treatment of malignant tumors. Now hundreds of publications have described the treatment of ^{90}Y glass microsphere[25]. The therapeutic practices in thousands of patients with liver cancer in about 80 medical centers around the globe show that hepatic arterial injection of ^{90}Y glass microsphere is a safe and effective method for liver cancer treatment. Carr reports a group of clinical control study involving 65 cases of primary liver cancer. Forty-two cases are Okuda stage 1 with a median survival of 649 d (244 d for the control group), 23 are Okuda stage 2 with a median survival of 302 d (64 d for the control group)[26]. Salem reports another clinical study involving 49 cases of liver cancer with a median survival of 85.9 mo. Twenty-eight patients reached the condition for tumor removal after the treatment and had their tumors removed, and 13 patients had tumor necrosis after the treatment. Their survival rate for 1, 3 and 5 years are 98%, 64% and 57%, respectively[27]. Intratumoral injection of ^{90}Y glass microsphere has also been studied. Tian reported that the tumor was reduced by 92% in 27 cases of primary liver cancer and 6 cases of metastatic liver cancer. α-fetoprotein in 13 cases recovered entirely after intratumoral injection[28]. These studies showed similar clinical therapeutic efficacy of ^{90}Y glass microsphere to that of surgical procedure. The ^{125}I-seeds used for prostate cancer in Europe and America has been tried in intratumoral implantation in China to treat advanced pancreatic carcinoma, which has demonstrated effects in alleviating pains and prolonging life[29-31]. In conclusion, directional radiotherapy using nuclide microsphere or nuclide particle is a potential alternative to treat malignant tumors in hepatic, biliary and pancreatic systems.

China is a country with a large population in the world and has the highest incidence and death rate of primary liver cancer. And the incidence of biliary and pancreatic malignant tumors is increasing. However, most of the patients with middle to advanced stage cancers of the three systems cannot be treated with surgery. Therefore, directional radiotherapy using nuclide microsphere will significantly improve the prognosis of patients with middle to advanced stage cancers. Since ^{90}Y glass microspheres have to be activated in an accelerator to get radioactivity, and when they are activated, they have relatively short half-life. We have been studying the nuclide microspheres for local brachytherapy for hepatic, biliary and pancreatic malignant tumors. In the 1990s, we developed the ^{32}P glass microsphere with relatively long half-life, and treated 40 patients with advanced liver cancer from 1992 to 1994 after the completion of the metabolism tests *in vivo* in tumor-carrying animals[32]. In recent years, we have developed the gelatin microspheres (GMSs) with a diameter of 50-70 μm carrying a high concentration of ^{131}I to treat patients with advanced liver cancer with hepatic arterial transfusion and embolotherapy. Since its half-life is 8.04 d and free ^{131}I in the body is either collected in thyroid tissue or discharged out of the body quickly, ^{131}I is safe and has relatively weak influence on other tissues[33-35]. And the GMS is one of the degradable biomaterials with good biocompatibility, which can also bind ^{131}I nuclide at a high concentration[36]. So in this study, we prepared ^{131}I-GMSs with a diameter of 10-30 μm for intratumoral implantation, which is expected to be easily applied for the hepatic, biliary, pancreatic and other malignant tumors that cannot be removed. Healthy rabbits are used as models to observe the metabolism of ^{131}I-GMSs *in vivo* and the tissue reaction in their livers.

MATERIALS AND METHODS

Materials

Lime-processed gelatin (sigma G-9382) with an isoelectric point of 4.8-5.2 was purchased from Sigma Co. Ltd., USA; ^{131}I-sodium-iodine solution (37 GBq/mL) was purchased from China Nuclear Group Chengdu Gaotong Isotope Co. Ltd., Chengdu, China. All other chemicals were of the highest commercially available purity.

Laboratory animals

This study was approved by the Animal Ethics Committee of Sichuan University. Twenty-eight healthy New Zealand rabbits weighing 1.8-2.5 kg were supplied by the animal experimental center of the Medical School of

Sichuan University and were divided into seven groups (groups 1-7), with four rabbits per group. Half of the rabbits were female and half were male. The rabbits were fed with a particulate (3-5 mm) chow and housed in a layered stainless steel coop. Rabbits had *ad libitum* access to running water. The air humidity and temperature were maintained at 50%-70% and 20-29℃, respectively. Eight days before the operation, four rabbits were randomly selected as control animals to collect serum from their hearts for the measurement of the liver and thyroid function.

Preparation of GMSs

GMSs were produced according to the modified method of Tabata *et al*[37]. Briefly, 10 mL of 10% lime-processed gelatin solution was added dropwise while stirring, to 80 mL of liquid paraffin (Kelong Chemical Reagent Co. Ltd., Chengdu, China), which was preheated to 55℃ with 0.8 mL span-80 (Shenyu Chemical Reagent Co. Ltd., Chongqing, China). The mixture was then stirred at 550 r/min at 55℃ for 15 min to yield a water-in-oil emulsion. The stirring was then continued for 30 min at 4℃. Next, 3 mL of glutaraldehyde (25%, Kermel Chemical Reagent Co. Ltd., Tianjin, China) was added to the mixture after it cooled for 5 min to induce crosslinking and solidification of the microspheres. The resulting microspheres were removed by suction filtration and washed three times with acetone (Changlian Chemical Reagent Industries, Ltd., Chengdu, China) after dehydration by immersion in 30 mL of acetone for 15 min. After air-drying, the GMSs were examined by the Analyzing and Testing Center of Sichuan University and imaged under a scanning electron microscope.

Preparation of ^{131}I-GMS

The ^{131}I was labeled by a modification of the chloramine-T method. Briefly, 50 mg of GMSs were placed in test tubes, rehydrated with 190 μL of phosphate-buffered saline (pH 7.0) for 10 min. Next, 3.4 μL of ^{131}I-sodium-iodine solution (37 GBq/mL) and 200 μL of chloramine-T solution (Bodi Chemical Reagent Co. Ltd., Tianjin, China) (20 mg/mL) were added to each test tube. After the reaction mixture had been incubated for 5-15 min at room temperature, with agitation, 200 μL of sodium pyrosulfite solution (Jiangbei Chemical Reagent Industries, Ltd., Wuhan, China) (15 mg/mL) was added to stop the reaction. The mixture was centrifuged (Eppendorf® 5702R refrigerated centrifuge, Eppendorf Co. Ltd., Hamburg, Germany) at 4400 r/min for 4 min to separate the ^{131}I-GMSs. Finally, the products were washed seven times with normal saline and sterilized by Co60 irradiation.

Implantation of ^{131}I-GMS in the liver

Each rabbit was injected intramuscularly with 0.1-0.2 mL/kg SuMianXin® (Veterinary Institute of the Chinese Military Academy of Medicine, Changchun, China) veterinary injection for anesthesia and placed on an animal operation table. The epigastrium was shaved, disinfected, and covered with a drape. The abdomen was opened with a 5-cm incision along the median line, and the left middle lobe or right middle lobe of the liver was fixed by the left index finger and thumb of the operator. Then, the mixture of 41.336 ± 5.106 MBq of the ^{131}I-GMSs, as measured in a Capintec® CRC-15R (Capintec, Inc., New Jersey, USA) dose calibrator, and 1 mL of 25% glucose solution (Kelun Pharmaceutical Industries, Ltd., Chengdu, China) drawn by a 1 mL syringe with a 4-G needle was slowly injected into the liver, after checking the needle was not inserted into a capillary. Two or three injections of 0.3-0.5 mL of the mixture were administered. When the injection points were not observed to be bleeding after being pressed for approximately 1 min after withdrawing the needle, the abdomen was closed and the operation was finished. The rabbit was immediately administered intramuscularly with 40 000 U gentamycin sulfate (Tianjin Pharmaceutical Co. Ltd., Tianjin, China).

Radioassay of rabbit serum

Four rabbits were chosen randomly for collecting 1 mL of blood from the ear veins between 0 and 24, then at 28, 32, 48 and 64 d after the administration of the GMSs. Those blood were centrifuged at 4400 r/min, and 250-μL aliquots of serum were used for γ counting in a γ counter (No. 262 Industry, Ltd., Xi'an, China).

Single-photon emission computed tomography, hepatic and thyroid functions examinations

The single-photon emission computed tomography (SPECT) scan (Skylight SPECT Camera, Philips Co. Ltd., Amsterdam, Netherlands) was conducted by the Nuclear Medicine Department of West China Hospital at 4 h, and at 1, 4, 8, 16, 24, 32, 48 and 64 d after administration. The animals in group 1, 2, 3, 4, 5, 6 and 7 were sacrificed at 1, 4, 16, 24, 32, 48 and 64 d after the surgery, respectively. Eight milliliters of blood was collected from each deceased animal. On day 8, four rabbits were randomly selected, and a further 8 mL of blood was collected from the heart of each rabbit on this day. Blood samples were used to assess hepatic and thyroid functions, which was conducted at the Biochemical Laboratory and the Hormonal Laboratory of the Experimental Medicine Department of West China Hospital.

Histological examination

Liver samples were fixed in 10% formalin solution (Kelong Chemical Reagent Co. Ltd., Chengdu, China) for 48 h. Liver samples were then embedded in paraffin, sectioned, and stained with hematoxylin and eosin (HE) for histological examination.

Statistical analysis

Results were expressed as mean ± SD and were analyzed by *t* tests using SPSS 11.5 software. The level of significance was regarded at a P value of < 0.05.

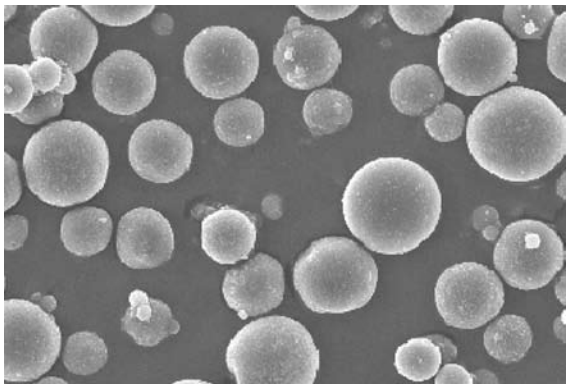


Figure 1 Scanning electron microscopic (20 kV) images of metal-coated gelatin microspheres (original magnification, × 500).

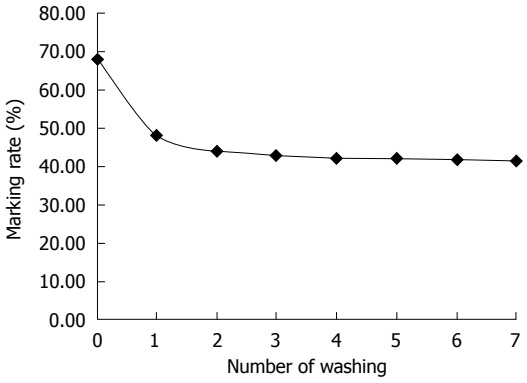


Figure 2 The labeling rate decreases with increasing the number of washes. The slope decreased gradually, nearly reaching a straight line, which demonstrates that the ^{131}I-labeled gelatin microspheres after washing contained very low amounts of nuclides conjugated by physical adsorption.

Table 1 Effect of washing on labeling rate (n = 6)

Number of washes	Labeling rate (%)
0	68.01 ± 2.09
1	47.74 ± 2.26
2	43.68 ± 2.19
3	42.72 ± 2.23
4	42.22 ± 2.27
5	41.91 ± 2.28
6	41.56 ± 2.27
7	41.00 ± 2.29

Table 2 Ratio of radioactivity between the liver and thyroid (n = 6) after the administration of ^{131}I-labeled gelatin microspheres

Time	Liver/thyroid
4 h	15.910 ± 0.740
Day 4	27.197 ± 5.467
Day 8	81.467 ± 24.637
Day 16	91.670 ± 23.278
Day 24	93.601 ± 21.337
Day 32	112.608 ± 12.787
Day 48	162.875 ± 7.955

RESULTS

Morphology of the microspheres

The GMSs were uniform in appearance, with a diameter of 10-30 μm, and a good divergence (Figure 1).

Effect of washing on GMS ^{131}I content

The GMSs labeled with ^{131}I were washed seven times to inhibit physical adsorption. Then, 50 mg of the GMSs labeled with ^{131}I showed decreased radioactivity levels with increasing number of washes. The slope for the relative ^{131}I content decreased gradually until it nearly reached a straight line (Table 1 and Figure 2).

Findings of SPECT imaging

The rabbits showed normal behaviors after the operation. SPECT imaging showed that the radioactive nuclide was concentrated in the liver, in regions surrounding the site of injection at 4 h and at 1, 4, 8, 16, 24, 32 and 48 d after ^{131}I-GMSs administration. However, SPECT did not reveal any nuclide labeling on day 64. The thyroid also showed low levels of nuclide accumulation on days 4, 8, 16 and 24. Furthermore, there was faint nuclide labeling in the bladder of four rabbits before day 24, although this disappeared by day 32. SPECT imaging showed no accumulation of nuclide in other tissues, including the lung, heart, stomach, intestines and kidney in any rabbits for the entire observation period (Figure 3). The radioactive ratios between the injected parts of liver and thyroids was assessed by region of interest analysis and increased with time, from 15.91 ± 0.74 at 4 h after ^{131}I-GMSs administration to 162.875 ± 7.955 at day 48 (Table 2).

Radioactive changes in serum

According to the serum γ counts, the radioactivity level decreased markedly over the first 2 d after ^{131}I-GMSs administration. The decline in radioactivity continued to decline thereafter, but at a slower rate until day 24. At this time, there was no difference in relative radioactivity level compared with the background level (Figure 4).

Hepatic and thyroid function

The levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) increased rapidly, particularly that of ALT, within 1 d after ^{131}I-GMSs administration. The levels of these enzymes then decreased gradually, reverting to the normal level by day 4. The values of alkaline phosphatase and γ-glutamyltransferase were relatively stable. Similarly, the total protein, albumin and globulin did not change markedly during the study period (Table 3 and Figure 5).

The levels of free triiodothyronine (FT3) and free thyroxin (FT4) were significantly decreased on day 8 (P < 0.05), but returned to normal levels at day 16, and remained at the normal level until day 64 (Table 4 and Figure 6). The level of thyrotropic-stimulating hormone (TSH) remained < 0.005 mU/L throughout the study.

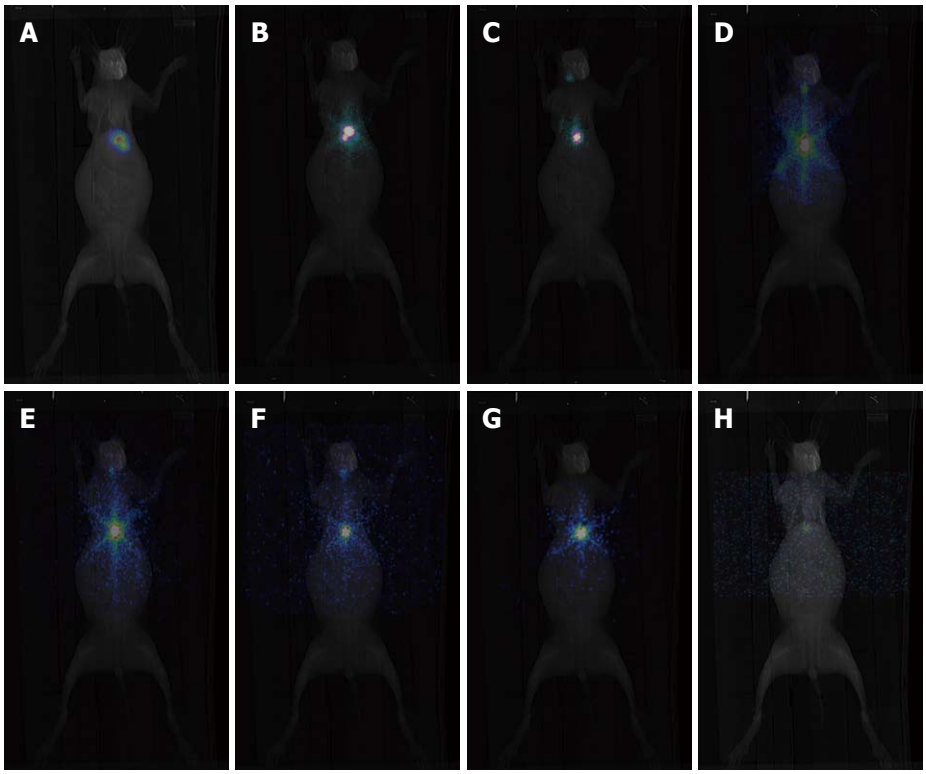


Figure 3 Single-photon emission computed tomography (SPECT) imaging performed at 4 h (A) and 1 (B), 4 (C), 8 (D), 16 (E), 24 (F), 32 (G) and 48 (H) d after the administration of ^{131}I-labeled gelatin microspheres.

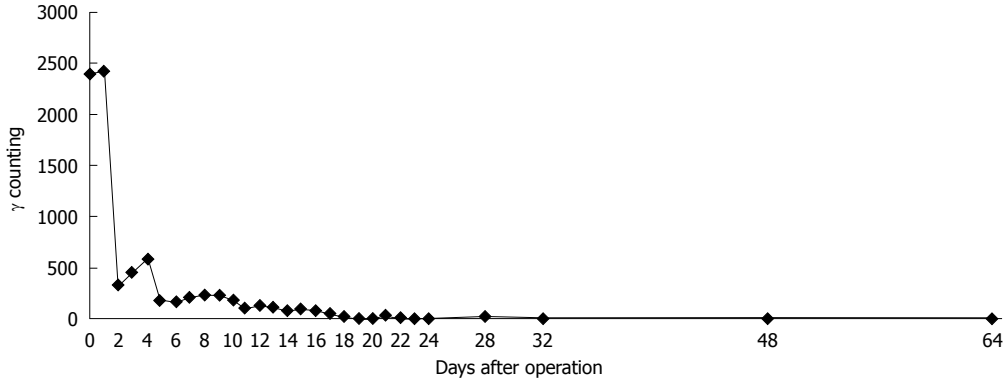


Figure 4 Dynamic changes in serum γ count after the administration of ^{131}I-labeled gelatin microspheres.

Table 3 Effects of the administration of ^{131}I-labeled gelatin microspheres on hepatic function (n = 4)

	ALT (IU/L)	AST (IU/L)	ALP (IU/L)	GGT (IU/L)	TP (g/L)	ALB (g/L)	GLB (g/L)
Before administration	67.75 ± 11.87	55.00 ± 11.87	99.75 ± 14.99	6.00 ± 1.83	54.33 ± 3.16	46.05 ± 2.32	8.28 ± 2.79
After administration							
1 d	291.50 ± 12.79	146.00 ± 13.88	115.00 ± 25.92	9.25 ± 3.30	60.23 ± 1.96	45.65 ± 5.17	15.00 ± 5.34
4 d	46.75 ± 4.79	41.25 ± 10.69	91.00 ± 29.59	7.25 ± 1.26	58.43 ± 8.45	47.55 ± 3.53	12.23 ± 5.03
8 d	48.50 ± 14.39	48.00 ± 17.32	79.75 ± 14.77	7.50 ± 0.58	58.10 ± 4.40	44.63 ± 2.33	13.65 ± 4.33
16 d	37.25 ± 9.32	39.75 ± 11.81	76.00 ± 9.83	9.50 ± 3.11	62.13 ± 5.84	44.38 ± 7.41	19.85 ± 6.79
24 d	46.50 ± 16.01	45.25 ± 9.95	96.25 ± 17.08	8.00 ± 2.83	60.28 ± 4.45	47.60 ± 7.38	14.50 ± 3.54
32 d	52.25 ± 14.29	46.25 ± 8.66	96.75 ± 35.58	12.50 ± 3.87	63.55 ± 4.90	46.40 ± 4.06	18.63 ± 6.72
48 d	43.75 ± 10.01	40.25 ± 14.45	97.25 ± 7.93	9.25 ± 1.71	60.78 ± 4.08	46.83 ± 4.06	14.23 ± 4.22
64 d	45.50 ± 4.80	45.75 ± 3.24	91.75 ± 29.62	10.50 ± 3.42	61.92 ± 8.18	50.15 ± 5.30	15.05 ± 3.89

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT: γ-glutamyltransferase; TP: Total protein; ALB: Albumin; GLB: Globulin.

Histological findings

The histological specimens showed that the ^{131}I-GMSs were quite concentrated, with a few inflammatory cells surrounding the injection sites on day 1 (Figure 7A) and some hepatic cells had died by day 4 (Figure 7B). Fibrous sheaths coating the ^{131}I-GMSs and sequential degradation of the ^{131}I-GMS were observed on days 16, 24 and 32. Most of the hepatic cells around and within the

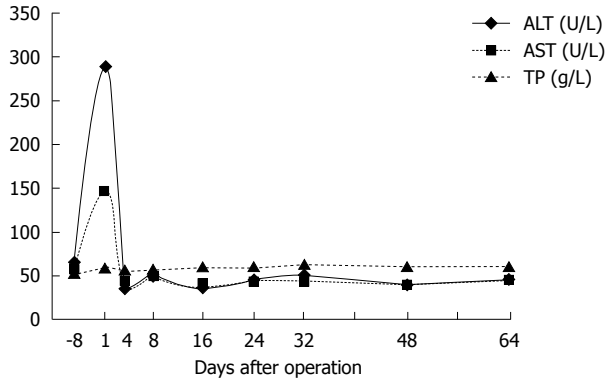


Figure 5 Changes in hepatic function after the administration of ^{131}I-labeled gelatin microspheres.

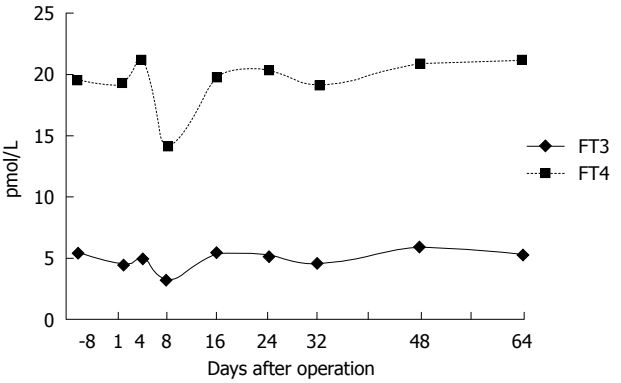


Figure 6 Curves of thyroid functional changes after the administration of ^{131}I-labeled gelatin microspheres.

Table 4 Effects of the administration of ^{131}I-labeled gelatin microspheres on thyroid function (n = 4)

	FT3 (pmol/L)	FT4 (pmol/L)	TSH (mU/L)
Before administration	5.55 ± 0.57	19.61 ± 1.54	< 0.005
After administration			
1 d	4.54 ± 1.53	19.31 ± 6.47	< 0.005
4 d	5.09 ± 1.68	21.31 ± 4.68	< 0.005
8 d	3.29 ± 0.83	14.23 ± 2.81	< 0.005
16 d	5.53 ± 1.01	19.95 ± 2.56	< 0.005
24 d	5.31 ± 0.57	20.39 ± 2.38	< 0.005
32 d	4.66 ± 2.17	19.18 ± 2.98	< 0.005
48 d	6.02 ± 2.77	20.94 ± 10.78	< 0.005
64 d	5.34 ± 1.34	21.23 ± 3.42	< 0.005

FT3: Free triiodothyronine; FT4: Free thyroxin; TSH: Thyrotropic-stimulating hormone.

sheaths died, leaving the hepatic cell cords on days 24 and 32. By day 24, the ^{131}I-GMSs had started to degrade and had an irregular shape (Figure 7C-E). However, the remnant ^{131}I-GMSs could still be histologically identified on day 48 (Figure 7F). No microspheres were found on day 64.

DISCUSSION

In recent years, many radioactive nuclide microspheres and nuclide particles have been successfully developed to target malignant tumors. However, because of the potential toxicity and side-effects associated with ^{90}Y, ^{32}P and other radioactive nuclides on marrow and other tissues, nonbiodegradable glass is often used as a carrier for most nuclides to avoid causing unwanted damage to normal tissues. However, glass has some limitations, including a protracted bioavailability due to its nonbiodegradable characteristics. Furthermore, its high specific gravity may adversely affect its injection and distribution. In this study, we generated degradable GMSs carrying the ^{131}I nuclide. Gelatin has a similar specific gravity to blood, and could be conjugated to many other drugs and nuclides *via* physical adsorption or chemical keys. Gelatin also shows good histocompatibility and degrades gradually *in vivo*[38-40]. Therefore, as a carrier for slow release of drugs, GMSs have been widely used by the medical community[41,42]. Meanwhile, ^{131}I is the most widely used radioactive nuclide in clinical settings, and has shown good anti-tumor effects in many clinical cases[43]. *In vivo*, dissociative ^{131}I mainly accumulates in the thyroid and is excreted *via* the kidney[44-46]. Therefore, ^{131}I that is released by the degradation of ^{131}I-GMS into the serum could result in tissue damage, particularly the thyroid gland. Therefore, detailed evaluation of the metabolic characteristics of ^{131}I-GMS, including its release, distribution and excretion *in vivo*, and the potential damage to the body should be evaluated to comprehensively appraise its safety. The study has provided some insight into these concerns.

The initial labeling rate of ^{131}I includes chemical binding and physical adsorption. When radionuclide-labeled microspheres are injected into the body, the nuclides conjugated to the microspheres *via* physical adsorption are more likely to dissociate. This has been reported to cause a severe de-iodinated state *in vivo*. In this study, we washed the microspheres seven times after the initial labeling, until the dissociative curve flattened, indicating that most of the ^{131}I conjugated *via* physical adsorption had been eluted and the ^{131}I-GMSs mainly exist *via* chemical combination. Although this reduces the labeling index, the *in vivo* de-iodination process is also attenuated, protecting against unwanted de-iodination effects.

Some studies have shown that injecting the microspheres at multiple sites could provide a more even distribution of the microspheres in the tumor tissues[47]. Therefore, in this study, we injected the microspheres in several sites in the liver. SPECT imaging revealed that the nuclides were principally localized to the injection sites at 4 h and at 1, 4, 8, 16, 24, 32 and 48 d after administration. The extrahepatic labeling with ^{131}I was mainly found in the thyroid gland between days 4 and 24 after administration, without any accumulation in other tissues, including the lung, heart, kidney, brain, stomach and intestines. Radioactivity of the serum could be detected for 24 d after the operation; after this time, the serum radioactivity level was not different to that of the background level. Because the thyroid gland is the

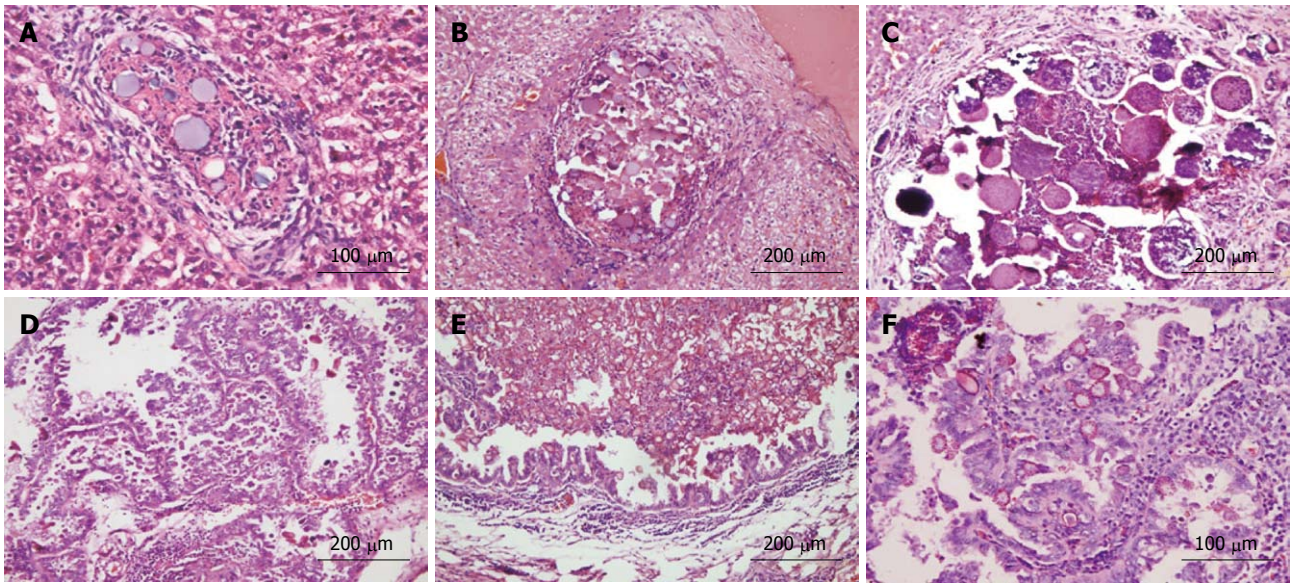


Figure 7 Pathological examination at 1 (A), 4 (B), 16 (C), 24 (D), 32 (E) and 48 (F) d after the administration of ^{131}I-labeled gelatin microspheres (HE stain; original magnification, × 200 in B, C, D and E, and × 400 in A and F).

principle site of iodine absorption and accumulation, it can absorb ^{131}I from the serum into thyroid follicles, which is shown on the SPECT scan. Based on the radioactivity ratio between the site of injection in the liver and the thyroid, it is clear that only a small amount of ^{131}I is absorbed by the thyroid.

SPECT imaging before day 24 revealed low radioactivity levels in the bladder, but not thereafter. This may be related to the full state of the rabbits' bladders when they were scanned, with full bladders showing some radioactivity as a result of excretion *via* the urinary system. Therefore, we believe that the release of ^{131}I from the microspheres mainly occurs within 24 d after administration, but only very small amounts are released.

Assessment of thyroid function revealed that the TSH level was consistently below 0.005 mU/L, while FT3 and FT4 declined on day 8, but returned to normal levels after day 16. This suggests that the thyroid is only subject to transient damage. Because the thyroid has its own repair mechanisms, the damage caused by some radiation doses can be repaired, without causing hypothyroidism[48]. In this study, a small amount of radioactive nuclides released into the blood was absorbed by the thyroid gland, but did not cause permanent damage or long-lasting hypothyroidism.

The assessment of hepatic function revealed that the ALT and AST increased rapidly compared with the normal level within 1 d after ^{131}I-GMSs administration. However, these parameters returned to the normal level 4 d later. This suggests that the administration of 41.336 MBq of ^{131}I caused notable liver damage in these experimental animals; however, because of the liver's capacity for self-repair and compensation, these impairments were transient and resolved within 4 d. This demonstrates the safety profile of radionuclide microspheres in the treatment of liver cancers.

From the pathological examination, we could conclude that the microspheres were gradually surrounded by fibroblasts to form fibrotic sheaths between days 16 and 24. This seemed to delay the degradation of the GMSs and reduced the rate of radionuclide release. This may explain the absence of thyroid radiolabelling from day 24 after surgery. Ohta reported that, after injecting GMSs into the renal artery of rabbits for 2 wk, the microspheres became wrapped with fibrous tissue and the GMSs in the embolism were completely biodegraded within 1 mo[49,50]. However, in this experiment, we used the intra-tissue implantation method, which differs from the arterial embolism method. Indeed, over four half-lives of ^{131}I decay (i.e. 32 d), the GMSs had degraded to varying degrees, but there was no sign of disappearance, with a large number of fiber-coated GMSs present, even by day 48. This difference may be due to the different methods of administering the GMSs. Previous studies have shown that gelatin is degraded in the body by degrading enzymes[51]. In this study, the ^{131}I-GMSs administered into the liver are, on the one hand, treated as a foreign body and induce foreign body reactions, with the activation of inflammatory cells, fibrotic cells and Kupffer cells to encapsulate and phagocytose the GMSs. On the other hand, the radiation will cause cells surrounding the GMSs to die to prevent phagocytosis by macrophages. This may explain why, in this experiment, the GMSs degrade slowly than that in the arterial embolism.

In conclusion, the hepatic administration of ^{131}I-GMSs in rabbits caused marked hepatic damage. Furthermore, there was some ^{131}I accumulation in thyroid tissue, causing slight, but only transient damage to the thyroid tissue. Other tissues showed no radioactive accumulation. Fibrous sheaths formed around the injected GMSs, which likely hampered the degradation of the

GMSs and protracted the release of ^{131}I. Taken together, we believe that it is safe to inject ^{131}I-GMSs into tissues *in vivo*, and these are likely to be effective against malignant tumors.

COMMENTS

Background

Malignant tumors in hepatic, biliary and pancreatic systems are very commonly encountered and treated surgically worldwide. In recent years, the incidence of malignant tumors in these three systems was reported to be rising worldwide. However, the rate of surgical removal of the malignant tumors of the three systems is very low and the prognosis after surgery is very unsatisfactory.

Research frontiers

Internal radiotherapy has become an important facet of clinical therapy for malignant tumors. ^{90}Y, ^{188}Re and ^{166}Ho microspheres have already been shown to be safe and are frequently used to therapy malignant tumors. However, their vehicle material is usually glass, which cannot degrade in the body. Unfortunately, once the nuclides have decayed completely, the vitreous carriers become foreign bodies that persist in the patient's body, and trigger foreign body reactions.

Innovations and breakthroughs

Nuclide microspheres and nuclide particles represent a new generation of tumor therapies and interventional radiotherapies. The authors used gelatin microspheres (GMSs) that can be easily produced to carry radionuclides, and can degrade *in vivo*. This study provides a foundation to show how nuclides attached to GMSs are distributed and metabolized *in vivo*. Healthy rabbits were used as a model to observe the metabolism of ^{131}I-GMSs. Overall, ^{131}I-GMSs were found safe for administration in rabbits.

Applications

By understanding the distribution and metabolism of ^{131}I-GMSs administered in the livers of rabbits, this study supports the use of ^{131}I-GMSs in directional internal radiotherapy for the treatment of hepatic malignant tumors. This approach could be applied to pancreatic, hepatic, biliary and other material malignant tumors that cannot be removed by surgery. This approach could also be considered to treat osteoarthritis.

Terminology

^{131}I-GMSs are protein microspheres containing the radionuclide ^{131}I. The half life of ^{131}I is 8.04 d and free ^{131}I either in the body or accumulates in the thyroid is quickly excreted *via* the urinary system. Compared with glass microspheres, the gelatin microsphere is a biodegradable material with good biocompatibility. In recent years, the authors have developed 50-70 μm nuclide protein microspheres that can be labeled with high concentrations of ^{131}I. These microspheres can be applied for hepatic arterial transfusion and embolotherapy for patients with advanced liver cancer.

Peer review

This is an interesting experimental study.The technique used in this study can be an effective method for treatment of maling tumors in the patients.

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BRIEF ARTICLE

Presence of hepcidin-25 in biological fluids: Bile, ascitic and pleural fluids

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Abstract

AIM: To examine body fluids such as ascitic fluid (AF), saliva, bile and pleural effusions for the presence of hepcidin using a novel radioimmunoassay (RIA).

METHODS: Serum samples were collected from 25 healthy volunteers (mean age: 36 ± 11.9 years, 11 males, 14 females). In addition bile was obtained from 12 patients undergoing endoscopic retrograde cholangiopancreatography (mean age: 66.9 ± 16.7 years, M:F = 5:7). Saliva was collected from 17 healthy volunteers (mean age: 35 ± 9.9 years, M:F = 8:9). Pleural and AF were collected from 11 and 16 patients [(mean age: 72 ± 20.5 years, M:F = 7:4) and (mean age: 67.32 ± 15.2 years, M:F = 12:4)], respectively. All biological fluid samples (serum, exudative and transudative fluids) were tested for the presence of hepcidin-25 molecule using RIA.

RESULTS: Hepcidin-25 was detected in all biological fluids tested. The mean ± SD hepcidin-25 in serum was 15.68 ± 15.7 ng/mL, bile 7.37 ± 7.4 ng/mL, saliva 3.4 ± 2.8 ng/mL, exudative fluid 65.64 ± 96.82 ng/mL and transudative fluid 14.1 ± 17.8 ng/mL.

CONCLUSION: We provide clear evidence that hepcidin-25 is present in bile, saliva, pleural and ascitic fluids. Hepcidin is likely to play a role here in innate immunity.

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Key words: Hepcidin; Hepcidin assay; Hepcidin in biological fluids; Hepcidin in ascitic fluid; Bile; Exudates; Antimicrobial peptides

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INTRODUCTION

Hepcidin-25 is a cysteine-rich circulating bioactive peptide that is predominantly secreted from the liver and excreted in the urine[1]. It is synthesized as preprohepcidin and undergoes posttranslational processing before release into circulation in an active form[2]. It is a central iron regulator with *in-vitro* evidence of antimicrobial properties. Hepcidin exerts its regulatory effect by preventing the efflux of intracellular iron by binding to ferroportin channels[3]. These ferroportin channels are present on the basal surface of the intestinal enterocytes as well as hepatocytes, macrophages and placental cells and are predominantly involved in the export of iron from these cells[4]. Despite a considerable body of literature on this novel peptide, research has been impeded by the lack of a reliable assay. Initially a semi quantitative assay (immunodot) was described but more recently quantitative assays have been developed using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS)[5] and liquid chromatography tandem MS (LC-MS/MS)[6,7].

We developed and validated a radioimmunoassay (RIA) for hepcidin-25 and measured serum levels of hepcidin-25 in various patient groups[8]. In this manuscript, we describe the use of RIA to demonstrate and measure hepcidin-25 in various biological fluids.

MATERIALS AND METHODS

This was a prospective study performed with approval of our Regional Ethics Committee and written consent was obtained from all patients and healthy volunteers in accordance with the Declaration of Helsinki. Patients and healthy volunteers were recruited from a single hospital with mixed ethnicity mainly comprising Caucasians and South Asians living in West London.

Healthy controls

The serum study comprised of 25 healthy controls (HC); 14 were females, mean age was 36 ± 11.9 years (age range: 21-62 years), were hospital colleagues recruited to measure serum hepcidin-25, ferritin and serum prohepcidin. HC with systemic illness (acute or chronic) or any history of regular use of medication including supplemental vitamins were excluded. A fasting venous blood sample was obtained between 08:30-09:00 h. Serum was separated and stored at -20℃ for analysis.

Bile

Bile was collected from 12 patients undergoing endoscopic retrograde cholangiopancreatography (ERCP) for proven or suspected choledocholithiasis, mean age 66.9 ± 16.7 years, M:F = 5:7 (age range: 40-88 years). All patients had ultrasonography (USG) of the abdomen to identify choledocholithiasis, or cholelithiasis. Ten out of 12 patients recruited had cholelithiasis confirmed on USG. Two patients had a dilated common bile duct (CBD) without choledocholithiasis. During ERCP, CBD was cannulated and 2 mL of bile was aspirated before injecting contrast medium for CBD visualisation. Stone extraction or biliary sludge removal including sphincterotomy was done after bile collection to avoid contamination. Bile obtained was stored at -20℃.

Saliva

Saliva was obtained from a separate cohort of 17 healthy volunteers, mean age of 35 ± 9.9 years (age range: 23-60 years), M:F = 8:9. A history of systemic illness or dental or pharyngeal pathology was excluded. Participants were not allowed to eat or drink 30 min before the collection of saliva. Ten minutes before collection, participants had to rinse their mouth with plain water. They had to chew on a cotton swab and return the same with saliva in sterile tube (Salivette, Sarstedt, UK) and stored at -20℃.

Pleural fluid

Eleven patients, mean age 72 ± 20.5 years (age range: 26-95 years), M:F = 7:4 were recruited for pleural fluid (PF) analysis. PF was collected by diagnostic thoracocentesis under sterile conditions and stored in a sterile container at -20℃. PF was analysed and divided into a transudate and exudate by comparing the total protein concentrations in fluid to that in blood as per Light's Criteria[9]. Three patients had transudative fluid (PF-T) (M:F = 2:1). The underlying cause for fluid accumulation in this group was congestive heart failure secondary to ischemic heart disease. Eight patients had exudative fluid (PF-E) (M:F = 5:3). The underlying cause for fluid accumulation in this cohort was parapneumonic (5 cases) and neoplastic [3 cases (1 breast and 2 lung malignancies)].

Ascitic fluid

Ascitic fluid (AF) was collected from 16 patients, mean age 67.32 ± 15.2 years (age range: 42-85 years), M:F = 12:4 under sterile conditions by diagnostic paracentesis as further differentiated by serum/ascites albumin gradient (SAAG)[10]. Twelve patients were identified as having transudative ascites (AF-T) (M:F = 8:4). Alcohol induced liver cirrhosis was the aetiology and comprised majority with 10 patients. The remaining 2 patients had chronic hepatitis B and chronic hepatitis C as etiology. The exudative ascites cohort comprised 2 patients (AF-E) (M:F = 2:0), with aetiology being congestive heart failure secondary to ischemic heart disease and idiopathic.

One patient had transudative AF with spontaneous bacterial peritonitis (SBP) (M:F = 1:0) analysed for presence of hepcidin-25. The cause of ascites was cirrhosis of the liver secondary to alcohol misuse. The AF white cell count was 350/mm^3 (90% polymorphs, 10% lymphocytes) and AF culture had grown *Escherichia coli* (*E. coli*).

On the basis of above, 2 cohorts were obtained, those with Transudative Fluid and those with Exudative Fluid, comprising both pleural and AFs in each respective category. One patient with SBP-ascites was excluded from the transudative fluid group. Thus, there were 10 patients

in the total exudative fluid cohort (M:F = 7:3) and 15 patients in the total transudative fluid cohort (M:F = 10:5).

Assay

Hepcidin-25 was measured using a novel competitive RIA. This was produced in our laboratory and has been described in our previous manuscript (Busbridge *et al*[8]).

The specificity of the antibody was also confirmed by Western blotting analysis of the precipitated proteins, where a single band of < 6 kDa was detected. Western blotting analysis was carried out by using 10 mL normal human plasma, which was first centrifuged and filtered through a 0.22 mm filter (Millipore, MA). After addition of 10-mL dithiothreitol, plasma was again filtered through a 30 kDa filter (Millipore) and the filtrate precipitated using 25% TCA. The precipitated proteins were resuspended in loading buffer and subjected to SDS electrophoresis on a 4%-16% NuPAGE Novex Bis/Tris gel (Invitrogen, UK). Western blotting was performed using an XCell Ⅱ blot module (Invitrogen) and a 0.1 mm pore size nitrocellulose membrane (Sigma Aldrich). Non-specific sites were blocked with 5% BSA in PBS and the membrane probed with purified rabbit anti-hepcidin antibody (1 in 2000 dilution in blocking buffer), followed by incubation with a secondary anti-rabbit antibody conjugated with horseradish peroxidase (Sigma Aldrich). Specific signals were detected with 3,3', 5,5' tetramethylbenzidine liquid substrate (Sigma Aldrich) (Figure 1).

Serum prohepcidin was measured by enzyme linked immunosorbent assay (ELISA) (DRG Diagnostics, UK) as per manufacturer's guidelines. Ferritin, iron and C-reactive protein were measured using Abbott Architect *i*8000 system (Abbott Diagnostics, Ireland).

Statistical analysis

Values are expressed as mean ± SD as indicated. Quantitative variables were compared using unpaired *t*-test; Pearson's rank correlation was used for calculating correlation between the hepcidin-25 and other variables. A value of $P < 0.05$ was considered significant. All statistical analyses were carried out using the statistical package GraphPad Prism version 5.0 for Windows, (GraphPad Software, San Diego California USA, http://www.graphpad.com).

RESULTS

HC

Serum hepcidin in HC was 15.30 ± 15.71 ng/mL with a range of 1.1-55.0 ng/mL. The mean prohepcidin was 236.88 ± 83.68 ng/mL and the mean ferritin was 110.00 ± 128.08 μg/L. There was no correlation between serum prohepcidin and ferritin in healthy volunteers. However, serum hepcidin showed a weak correlation with ferritin ($r = 0.174$, $P < 0.05$).

Bile and saliva cohort

Hepcidin-25 was detected in all bile samples ($n = 12$) and was measured at a mean of 7.37 ± 7.39 ng/mL with a range of < 1.0-23 ng/mL. Hepcidin-25 was detected in saliva with a mean of 3.39 ± 2.83 ng/mL, range of < 0.9-11.8 ng/mL.

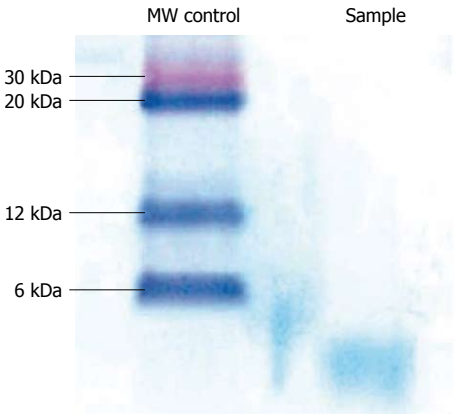


Figure 1 Western blotting analysis of serum proteins using rabbit anti-hepcidin antibody demonstrating the presence of a protein band at < 6 kDa.

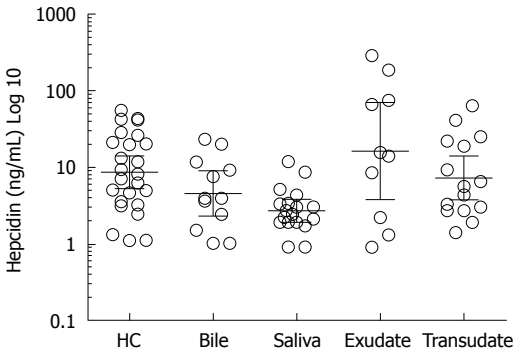


Figure 2 Scatter-dot plot of hepcidin data from healthy control sera (HC) and various body fluids, bile, saliva and pleural fluid both exudates and transudate in nature. Data is presented with geometric mean and 95% CI levels.

Exudative and transudative fluid

Hepcidin-25 level was 65.64 ± 96.82 ng/mL in exudative fluid. The mean total protein level in the fluid was 43.69 g/L. The hepcidin-25 level was 14.10 ± 17.88 ng/mL in transudative fluid. Hepcidin-25 concentration in exudative fluid was higher than in transudative but did not reach statistical significance ($P = 0.05$, student *t*-test). This could be due to the small number of patients involved in the study. We demonstrated hepcidin-25 in all bodily fluids analysed using RIA. To our knowledge this is the first report confirming the presence of hepcidin-25 in these bodily fluids (Figure 2).

DISCUSSION

Hepcidin, as an antimicrobial peptide, was first described as a liver expressed antimicrobial peptide 1[11].

Hepcidin is a novel peptide due to its dual role as an 'iron-hormone' and 'antimicrobial peptide'. However, previous research studies have been hampered due to lack

of a reliable quantitative assay for hepcidin-25. Several factors have been cited for this difficulty including presence of non-biologically active iso-forms of hepcidin-20, hepcidin-22, and the fact that hepcidin-25 is conserved over evolution thus making an immunological response in host species difficult to determine[12]. Several semi quantitative and quantitative assays have recently been developed for hepcidin-25 measurement. The quantitative assays include ELISA[13] and MS. MS methods includes TOF-MS, LC-MS, matrix-assisted laser desorption ionization MS, and SELDI-MS[14,15]. Although very successful, the availability of the MS assay is limited to specialist laboratories as they require strict internal standards and specialised equipment. Two quantitative assays have been described using ELISA techniques. Ganz *et al*[13] have described a competitive quantitative ELISA assay using functional synthetic peptide hepcidin-25 while the method described by Koliaraki *et al*[16] detects hepcidin-25 using a recombinant functional hepcidin-25 peptide. Thus, although a number of methods are available to measure hepcidin-25, there does not appear to be a standardisation amongst various methods. The results of the first international round robin for the quantification of urinary and plasma hepcidin assays were published in an attempt to help standardisation issues[17].

Until recently, hepcidin-25 was shown to be present in urine and serum. However, its expression by various organs such as salivary glands, tonsils, trachea, lung and prostate have also been reported[18]. The exact nature and function of hepcidin expression in these organs remains unclear.

Hepcidin in saliva

This study confirms the presence of hepcidin-25 in saliva. Its exact role in saliva is still uncertain but could contribute to the broad spectrum defence of the oral cavity along with peptides such as cathelicidins[19].

Hepcidin in bile

The presence of hepcidin-25 in bile may be a mechanism of hepcidin-25 excretion apart from urine. It may act along with α-defensins against gut microflora[20]. Further studies are needed to fully understand its role in bile.

Hepcidin in PF and AF

We were able to demonstrate the presence of hepcidin in pleural and AF where its iron regulator function is hard to explain. Hepcidin present in these fluids has unknown biological activity. Hepcidin-25 is a cationic peptide with 4 disulfide bridges[21]. The connection between hepcidin and infection/inflammation became clearer after the initial discovery in 2000. Shike *et al*[22], in 2002 showed that in white bass liver, infection with the fish pathogen *Streptococcus iniae* increased hepcidin mRNA expression 4500-fold. In another study by Nicolas *et al*[23] in 2002 injections of turpentine, a standard inflammatory stimulus, into mice induced hepcidin mRNA 4-fold and led to a 2-fold decrease in serum iron.

Hepcidin maintains a role in host defence by acting as a bridge between immunity and iron metabolism, as indicated by markedly induced hepcidin by infection and inflammation[24-26]. Park *et al*[1] have shown that hepcidin has antimicrobial activity against *E. coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and group B *Streptococcus*. Hepcidin also exhibits antifungal activity against *Candida albicans, Aspergillus niger* and *Aspergillus fumigatus*.

Our study on the presence of hepcidin in bile, saliva and various other biological fluids provides further confirmatory evidence that hepcidin has evolved from being an antimicrobial peptide. Hepcidin may form part of human innate immunity and complement other antimicrobial peptides to provide a wide spectrum of mucosal immunity against pathogens.

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COMMENTS

Background

Until recently iron metabolism had been extensively studied but poorly understood. With the discovery of hepcidin as an iron regulator in the body, there has been a significant growth in the knowledge related to iron metabolism. Crucial research has been done to explore the role of hepcidin in iron deficiency anaemia, anaemia of chronic disease and iron excess disorders.

Research frontiers

Hepcidin is a 25 amino-acid peptide predominantly formed in the liver and is a key component in iron homeostasis. Its major role is to prevent iron export by binding to iron export channels, ferroportin, leading to internalisation and lysosomal degradation of the hepcidin-ferroportin complex. Interestingly, hepcidin is found throughout evolution and in amphibian species has possible antimicrobial properties while in humans its major action is iron metabolism. Prior research showed hepcidin expression in various human organs like heart, intestine and salivary glands. In this study, the authors demonstrate clear evidence that hepcidin is present in various bodily fluids with higher levels found in exudative fluids and could be a potential mechanism for innate immunity preserved over evolution.

Innovations and breakthroughs

Recent studies have highlighted the importance of hepcidin in iron metabolism, particularly anaemia of chronic disease and iron overload. There have also been reports of its expression in various proinflammatory disorders and various organs, linking it to innate immunity and iron metabolism. This is the first study to report that hepcidin is found in various biological fluids. Furthermore, *in-vitro* studies have suggested that hepcidin has antimicrobial activity.

Applications

By understanding how hepcidin is expressed and by blocking its expression, there may be a therapeutic potential in patients with anaemia of chronic inflammation. Further application could be that secondary infection of pleural and ascitic fluid complicating underlying diseases may be better understood by studying hepcidin levels in infected and non-infected patients.

Terminology

Hepcidin is a protein involved in iron metabolism. Hepcidin is formed predominantly in the liver and is released in the circulation where its major function is hypoferremia by binding to iron export channel ferroportin. Hepcidin is expressed by various organs and thus it was conceivable that hepcidin would be found in various biological fluids. The exact role of hepcidin in these fluids remains uncertain and more studies are needed.

Peer review

The authors present a nicely conceived and analysed study. It is well written and offers new information about the presence of hepcidin in human biological fluids.

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BRIEF ARTICLE

Probiotic yeasts: Anti-inflammatory potential of various non-pathogenic strains in experimental colitis in mice

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Abstract

AIM: To evaluate the *in vitro* immunomodulation capacity of various non-pathogenic yeast strains and to investigate the ability of some of these food grade yeasts to prevent experimental colitis in mice.

METHODS: *In vitro* immunomodulation was assessed by measuring cytokines [interleukin (IL)-12p70, IL-10, tumor necrosis factor and interferon γ] released by human peripheral blood mononuclear cells after 24 h stimulation with 6 live yeast strains (*Saccharomyces ssp.*) and with bacterial reference strains. A murine model of acute 2-4-6-trinitrobenzene sulfonic acid (TNBS)-colitis was next used to evaluate the distinct prophylactic protective capacities of three yeast strains compared with the performance of prednisolone treatment.

RESULTS: The six yeast strains all showed similar non-discriminating anti-inflammatory potential when tested on immunocompetent cells *in vitro*. However, although they exhibited similar colonization patterns *in vivo*, some yeast strains showed significant anti-inflammatory activities in the TNBS-induced colitis model, whereas others had weaker or no preventive effect at all, as evidenced by colitis markers (body-weight loss, macroscopic and histological scores, myeloperoxidase activities and blood inflammatory markers).

CONCLUSION: A careful selection of strains is required among the biodiversity of yeasts for specific clinical studies, including applications in inflammatory bowel disease and other therapeutic uses.

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Key words: Yeast; Probiotics; Strain specificity; Experimental colitis; 2-4-6-trinitrobenzene sulfonic acid

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INTRODUCTION

Yeasts, as an inevitable part of the microflora of various fermented foods and beverages, are found in a wide range of foods from plant or animal origin, where they have a significant impact on food safety and organoleptic properties. Both baker's and brewer's yeasts [*Saccharomyces cerevisiae* (*S. cerevisiae*)] have been available for the last

decades as dietary supplements because of their high contents of vitamin B, proteins, peptides, amino-acids and trace minerals. Regardless of their non-human origin, such non-pathogenic yeasts fulfill the major criteria for probiotic definition[1]. Interest in probiotic yeasts has increased, especially in relation to animal feed but also for human applications. Hence, yeasts are rarely (if ever) associated with outbreaks or cases of food-borne illness and, through their history, many yeast species are recognized as safe, as confirmed by their Qualified Presumption of Safety status, assigned by the European Food Safety Authority (http://www.efsa.europa.eu). However, some rare cases of *Saccharomyces boulardii* (*S. boulardii*) fungemia have been reported, although they are essentially restricted to immunocompromised patients or associated with patients being contaminated through a central venous catheter[2]. Also, identification of the contaminating yeast strains in some cases could be questioned, as it was often based on cross-reacting anti-yeast mannan circulating antibodies, shared with *Candida* species.

Although *S. boulardii* was originally selected using rather empiric methods, this yeast species has many proven benefits in various gastrointestinal diseases[3-5] and is well recognized as the non-bacterial prototype of a probiotic. Multiple mechanisms have been suggested that explain the broad health-promoting effects of consuming food grade yeasts, ranging from local general trophic effects to action on both innate and/or adaptive immunity[6,7]. The yeast-induced probiotic signals might be either prophylactic or therapeutic in a defined pathologic context or in a specific application.

Most reported effects of yeasts as probiotic organisms in clinical trials stand for alleviation of (1) antibiotic-associated diarrhea; (2) infectious diarrhea [including recurrent *Clostridium difficile* (*C. difficile)* related diseases, CDAD]; (3) irritable bowel syndrome; and (4) inflammatory bowel diseases (IBD). Focusing on IBD, *S. boulardii* efficacy was both observed in Crohn's disease[8] and by favoring remission of ulcerative colitis[9]. Most of the therapeutic approaches in IBD were also using anti-inflammatory drugs such as mesalamine concomitantly and the exclusive use of yeast as possible ecological treatment has not been tested, most probably for ethical reasons. The anti-inflammatory potential of yeasts has been addressed by many *in vitro* studies elaborating on multiple mechanisms[4,7]. Surprisingly, data using yeasts in animal models of colitis are very scarce and the precise mechanism(s) of action by which such intervention may exert its beneficial effects *in vivo* is consequently poorly documented. Dalmasso *et al*[10] have reported a beneficial action of secreted yeast molecules on T cell migration in the chronic model of CD4+CD45RB^{hi} T-lymphocytes transfer into SCID mice. The same authors have also reported the preventive effect of *S. boulardii* in an acute murine 2-4-6-trinitrobenzene sulfonic acid (TNBS) model, characterized by a strong inhibitory activation of NFκB and the reduction of mRNA for pro-inflammatory cytokines together with decreased histological scores of inflammation[11,12]. Consistently, a similar TNBS-induced model of colitis, although developed for a more therapeutic approach in rats, also showed appreciable reduction of histological scores and pro-inflammatory colonic markers by *S. boulardii* (including cytokines but also metabolites of pro-oxidative pathways such as iNOS). The proposed mechanism depended on PPAR activation by the probiotic yeast[13], a regulator previously demonstrated to reduce intestinal inflammation[14]. Finally, yeast-driven therapy in a DSS-induced colitis model was also successfully demonstrated in mice[15], even when this chemically induced colitis was amplified by *Candida albicans* colonization. Because the model is known to affect epithelial crypt cells and destroy barrier integrity without intervention of the adaptive immune system[16], it was suggested that the contribution of yeast-mediated protection was restricted to the switching off of the innate mechanisms of colitis and/or to the enhancement of the mucosal barrier integrity.

Very few yeast strains have been studied as possible biotherapeutics agents and, at the moment, *S. boulardii* is the only yeast commercialized for human use, and consequently the single preparation formally recognized as probiotic. However, other *Saccharomyces spp.* or members of other yeast genera with similar or possibly better therapeutic properties will certainly be isolated in the future[17,18]. Hence, distinct non pathogenic yeast strains may have their own impact on gut homeostasis, sharing some of the common mechanisms of *S. boulardii,* but also other more specific consequences or even novel prophylactic or therapeutic effects.

Considering the widespread use of lactic acid bacteria and bifidobacteria as probiotics, it is now well accepted that all strains are not equally beneficial, that each may have individual mechanisms of action. Furthermore, host characteristics (e.g. flora composition or immune status) may determine which probiotic species or strains may be optimal[19]. Peran *et al*[19] conclude that it would be interesting to compare different probiotics in the same experimental model, in order to establish the best profile in a given setting. Likewise, this reasoning can be extent towards "friendly yeasts" as far as evaluation is conducted for a specific use without generalizations about probiotic effects[20,21].

In this paper, we compared yeast strains in their ability to induce cytokines on human peripheral blood mononuclear cells (PBMC). Our results also demonstrated that some yeast strains exhibit significant anti-inflammatory activities *in vivo*; whereas other may have weaker inflammation lowering effects or no anti-inflammatory effect at all. Thus, as for probiotic bacteria[20], strain-specific differences were seen for yeasts, suggesting that careful selection of strains for therapeutic use is required for preclinical and further clinical studies, including for applications in IBD.

MATERIALS AND METHODS

Microbial strain preparation and growth conditions

All *Saccharomyces* strains used in this study and listed (Table 1), including baker's yeast *S. boulardii* and wine-

Table 1 Bacterial and yeast strains and their origin used in the present study

Strain	Identification	Origin
BB536	*Bifidobacterium longum*	Morinaga Milk Industry Ltd.
Ls33	*Lactobacillus salivarius*	Commercial strain/ Danisco
NCFM	*Lactobacillus acidophilus*	Commercial strain/ Danisco
MG1363	*Lactococcus lactis*	Cheese starter[22]
TG1	*Escherichia coli*	Commensal strain[23]
LV01/ CNCM I-3799	*Saccharomyces boulardii*	Lesaffre collection
LV02/ CNCM I-3856	*Saccharomyces cerevisiae*	Lesaffre collection
LV03	*Saccharomyces pastorianus*	Lesaffre collection
LV04	*Saccharomyces bayanus*	Lesaffre collection
LV06	*Saccharomyces cerevisiae*	Lesaffre collection
LV09	*Saccharomyces cerevisiae*	Lesaffre collection

CNCM: French National deposit Collection of Microorganism Cultures, Institut Pasteur, Paris, France.

related strains, were originally isolated from a proprietary germoplasm bank of *S. cerevisiae* (Societe Industrielle Lesaffre 147 Rue Gabriel Peri, BP 6027, 59700 Marcq-en-Baroeul Cedex, France). They were all provided by Lesaffre as a similar dry form with a cell concentration near to 1×10^{10} colony-forming units (CFU)/g. The different dried yeast products were processed and enumerated as follows. For both *in vitro* and *in vivo* assays, yeast was rehydrated with phosphate buffered saline (PBS) (pH 7.2) 1:10 (w/v) at room temperature, and adjusted to an appropriate concentration in CFU/mL. For this purpose, cellular counts together with viability, expressed in %, were routinely performed using a hematimeter (type Thoma) combined with a Trypan blue exclusion method. More than 95% viability was preserved following rehydration. Enumeration was also confirmed *a posteriori* by plating on *YPD-agar* and allowing growth during 2 d at 30℃ and 37℃.

Some bacterial strains were used as reference strains for immune cell stimulation as previously described[24], also listed (Table 1). *Lactobacillus* strains were grown under limited aeration at 37℃ in MRS medium (Difco) and a *Bifidobacterium* strain was grown anaerobically in MRS supplemented with 0.05% L-cysteine-hydrochloride (Sigma). *Lactococcus lactis* MG1363 was grown at 30℃ in M17 medium supplemented with 0.5% glucose. *Escherichia coli* (*E. coli*) was grown at 37℃ in LB medium (Difco). Bacterial cells were grown until stationary phase, washed and resuspended at 1×10^9 CFU/mL in PBS containing 20% glycerol and stored at -80℃ until required for later assays. Some routine analyses were also performed using a portable photometer (Densimat bioMerieux)[25]. Cells were adjusted to McFarland 3 and stored at -80℃.

In vivo resistance to gastrointestinal tract

Groups contained 10 mice. When needed, fecal samples from 5 mice were collected, pooled, weighed and mechanically homogenized in sterile neutral and isotonic buffer at 50 mg/mL of feces. Dilutions were plated onto the "yeast-selective" tetracycline (150 μg/mL) *YPD-agar* (Yeast extract, Peptone, Dextrose, as described elsewhere[26]. No tetracycline-resistant microorganisms (bacteria or fungi) were detected in non-inoculated mice, indicating no or negligible basal colonization of mice gastrointestinal tract (GIT) by non-pathogenic yeast nor *Candida* species.

PBMC isolation

PBMCs were isolated from peripheral blood of healthy donors as previously described[24]. Briefly, after a Ficoll gradient centrifugation (Pharmacia, Uppsala, Sweden), mononuclear cells were collected, washed in RPMI 1640 medium (Live technologies, Paisley, Scotland) and adjusted to 2×10^6 cells/mL supplemented with gentamicin (150 g/mL), L-glutamine (2 mmol/L), and 10% foetal calf serum (Gibco-BRL) supplementation.

Induction of cytokine release

PBMC (2×10^6 cells/mL) were seeded in 24-well tissue culture plates (Corning, NY). Twenty microliters of the thawed bacterial or yeast suspensions at 10^9 CFU/mL in PBS containing 20% glycerol (bacteria:cell or yeast: cell ratio of 10:1) or standardized at homogeneous cell density, were added. PBS containing 20% glycerol was used as a negative (non-stimulated) control. On the basis of preliminary time-course studies, 24 h stimulation corresponded to the best time point for cytokine responses of bacteria stimulated-PBMCs. After 24 h stimulation at 37℃ in an atmosphere of air with 5% CO_2, culture supernatants were collected, clarified by centrifugation and stored at -20℃ until cytokine analysis. Concerning yeast stimulation, freshly rehydrated powders were used at distinct yeast:cell ratios (from 0.1:1 to 5:1), with or without the presence of fungizone (amphotericin B, 10 μg/mL). Neither medium acidification nor bacterial or fungal proliferation was observed. Cytokines were measured by enzyme-linked immunosorbent assay (ELISA) using BD Pharmingen antibody pairs (BD Biosciences, San Jose, Ca, USA) for interleukin (IL)-10, interferon γ (IFNγ) and IL-12p70, and R&D systems (Minneapolis, Mn, USA) for human tumor necrosis factor (TNF), according to the manufacturer's recommendations.

Induction of colitis and inflammation scoring

BALB/c mice (female, 7 to 8 wk old) were obtained from Charles River (St Germain sur l'Arbresle, France). A standardized murine TNBS colitis model was used in which moderate levels of inflammation were induced[27]. Briefly, a 50 μL solution of 100 mg/kg TNBS (Sigma) in 50 % ethanol was slowly administered in the colon *via* a 3.5 F catheter. Freshly rehydrated yeast suspensions (100 μL), containing 1×10^9 CFU/mL in $NaHCO_3$ buffer were administered intragastrically to mice each day, starting 4 d before until the day of TNBS administration while control mice received the corresponding buffer. Mice were weighed, bled from the retro-orbital venous

plexus and killed 72 h after TNBS administration. Cleared sera were frozen and stored until cytokine assays at -20℃. Colons were removed, washed and opened longitudinally. Inflammation grading was performed by two blinded observers, using the Wallace scoring method[28]. Results are expressed as % protection, corresponding to the reduction of the mean macroscopic inflammation score of treated mice (n = 10) in comparison to the mean score of TNBS-treated control mice ($NaHCO_3$ buffer-treated mice, n = 10)[27]. Histological analysis was performed on May Grünwald-Giemsa stained 5 μm tissue sections from colon samples fixed in 10% formalin and embedded in paraffin and tissue lesions were scored according to the Ameho criteria[29]. Additionally, the degree of polymorphonuclear neutrophil infiltration of the distal colon was assessed by quantifying myeloperoxidase (MPO) - a neutrophil granule enzyme - as reported earlier[30]. A commercial preparation of prednisone (Cortancyl, Sanofi Aventis, France) was used as a positive control of protection and was orally administered for 2 subsequent days at 10 mg/kg starting at the day before TNBS administration. When needed, heparinized whole blood was collected by retro-orbital puncture and, after separation, mice sera samples were stored at -20℃ for subsequent analysis.

Cytokine and serum amyloid A protein assays

Murine IL-6 and serum amyloid A (SAA) protein levels were measured by ELISA using commercial antibodies from Pharmingen antibody pairs (BD Biosciences, San Jose, Ca, USA), and Biosource International (Camarillo, Ca, USA), respectively, with a lower limit of sensitivity of 15 pg/mL for IL-6 and 30 ng/mL for SAA.

Statistical analysis

All analyses were performed as comparison of experimental groups with respective controls by the non-parametric one-way analysis of variance Mann-Whitney U-testing, or by Student-T testing where appropriate. Differences were judged to be statistically significant when the P value was < 0.05. Data are presented as mean ± SE.

Ethical considerations

All experiments were performed in an accredited establishment (number 59-35009; Institut Pasteur de Lille, France) and using approved guidelines according to French Ethical Committee and European Union Normatives (number 86/609/CEE).

RESULTS

Production of cytokines by human PBMCs in response to food-grade yeast strains

To determine the optimal dose of yeast that can induce cytokine production in human PBMCs, immuno-competent cells were first stimulated with three different multiplicity levels of infection (0.1:1, 1:1 and 5:1) of three live yeast strains and compared with immuno-stimulatory effects of reference bacteria (ranging from weak to strong inducers). Substantial amounts of IL-10, and to a lesser extent for TNF were dose-dependently induced by yeast cells (Figure 1A and B, respectively) while no or very low levels of the pro-inflammatory indicators IL-12 p70 and IFNγ could be detected following yeast stimulation (Figure 1C and D). Following these results, the yeast: host cell ratio of 1:1 was retained for further comparative analyses between strains in order to mimic physiological interactions between cells but without saturating the stimulatory capacity. In addition, the variable responsiveness due to individual blood donors was highly minimized by expressing data as % of a reference strain instead of in pg/mL, as shown (Figure 2, panel A and B). PBMCs from 5 donors were stimulated with each of the six yeast strains covering distinct subspecies and origins (Table 1).

All individual strains of yeast induced relatively high levels of the anti-inflammatory IL-10, reaching the values generally obtained with lactobacilli (mean/max values 535/2300 pg/mL). However, in contrast to bacteria, no significant strain-specificity was observed among different strains of yeasts, which all induced similar levels of cytokines (Figure 3A). Analysis of the cell culture supernatants revealed moderate induction of TNF after yeast stimulation, as compared with bacterial stimulation, again without a clear strain dependency (Figure 3B). Yeasts induced extremely low levels of the pro-inflammatory signals IL-12 and IFNγ as compared to some bacteria such as *Lactococcus lactis* MG1363 or *Lactobacillus acidophilus* NCFM (Figure 3C and D). Concerning the intrinsic immunostimulatory capacity of yeast cells *in vitro*, we can conclude that no specific stimulation pattern could be discriminated among the six strains, while all strains favored anti-inflammatory profiles with a high IL-10/IL-12 ratio, low induction of TNF and negligible levels of IFNγ.

Resistance of various yeasts strains to the GIT passage

Next to the effect of yeasts on cytokine induction on PBMCs *in vitro*, we compared the ability of the 3 strains LV01, LV02 and LV09 to survive the digestive tract of mice. Following 0, 1 and 5 consecutive days of intragastric feeding of 10^8 CFU of each of the yeasts; faecal enumeration showed equivalent survival counts for all the strains with a progressive increase with the duration of the treatment (Figure 4). The systematic increase by 2 log units between day 1 and day 5 suggests that proliferation occurs in the gut, rather than simple transit and shedding. Although we cannot speculate on the long term persistence and colonization of the yeast species, a 5-d-treatment clearly leads to a noticeable presence of living yeasts in the colon lumen (up to 10^8 CFU/g for the 3 strains). A significant faecal recovery of yeast colonies from healthy mice was still detectable 10 d after the last feeding (more than 10^5 CFU/g, data not shown). It is noteworthy to mention that neither diarrhoea nor slight changes in faecal character, nor weight disturbance or other deleterious signs were observed in mice treated with those food-grade yeasts.

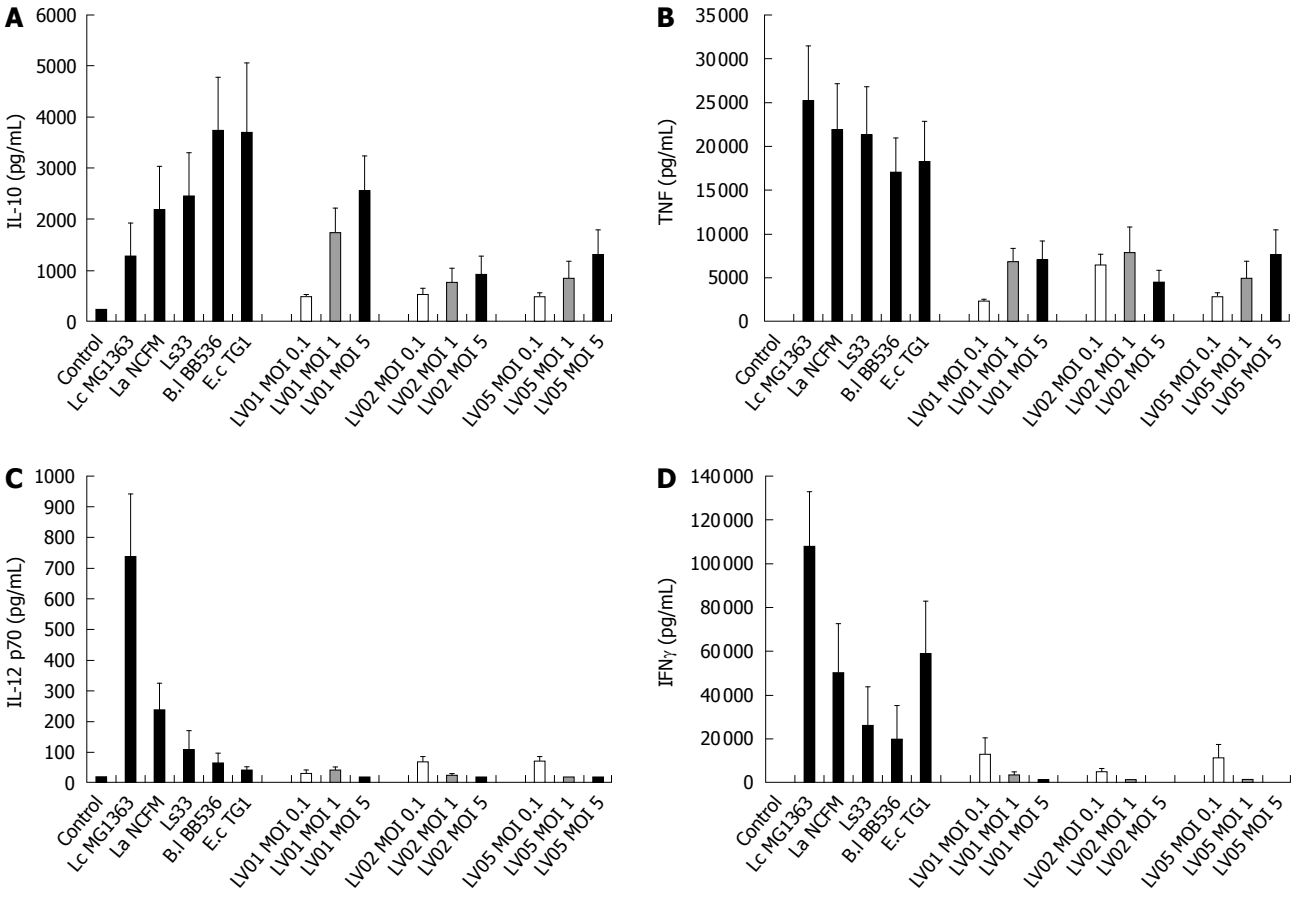


Figure 1 Dose-dependent cytokine production in human peripheral blood mononuclear cells (PBMCs), stimulated with bacterial reference strains and 3 yeast strains at distinct multiplicity levels of infection [multiplicity levels of infection (MOI) 0.1, 1 and 5 yeasts/cells] for 24 h. Cytokine levels were analyzed in supernatants by enzyme-linked immunosorbent assay (ELISA). Panels A, B, C and D respectively show results for interleukin (IL)-10, tumor necrosis factor (TNF), IL-12 p70 and interferon γ (IFNγ), respectively. Results are expressed in pg/mL, mean ± SE, *n* = 3 distinct donors.

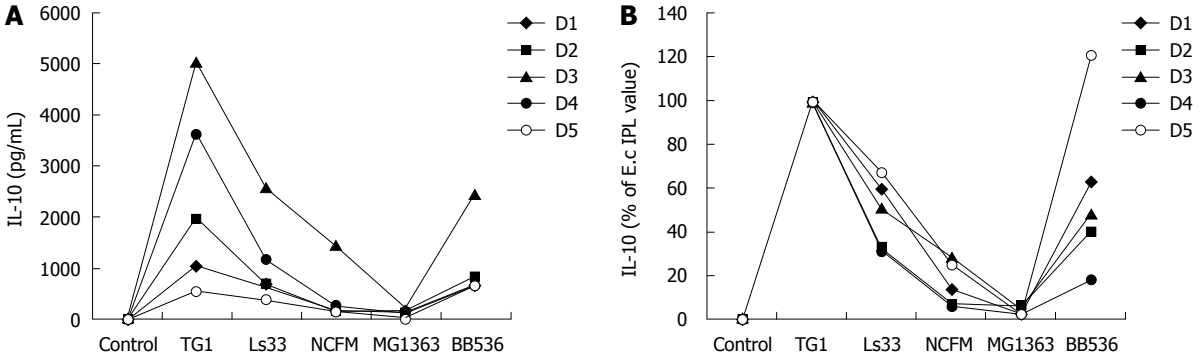


Figure 2 Standardized assessment of cytokine production in human PBMCs from distinct donors. Immuno-competent cells are stimulated with 5 bacterial reference strains for 24 h and cytokine levels were analyzed in supernatants by ELISA. A: IL-10 release for 5 individual donors. Results are expressed in pg/mL; B: Standardized IL-10 release with values expressed as % of *Escherichia coli* (*E. coli*) value for each of the 5 donors.

Comparative preventive capacity of yeasts in alleviating experimental colitis

In order to evaluate whether the administration of distinct yeast strains equally impacted on TNBS-induced colitis, we compared the performance of the three strains LV01, LV02 and LV09 with the anti-inflammatory corticoid drug (prednisone) which we and others previously used in this experimental setting[31,32]. Since some anti-inflammatory effects of lactobacilli were observed both after oral and systemic administration[33,34], we assessed whether intraperitoneal administration of the yeast LV01 could also rescue mice from colitis.

Rectal administration of TNBS causes progressive weight loss in vehicle-treated mice, reaching up to 22.5% of the initial weight 72 h after induction of colitis (Figure 5A and B). As expected, prednisone prevented body weight loss associated with experimental colitis (-3.3%, $P < 0.001$). The oral treatments by the 3 yeast

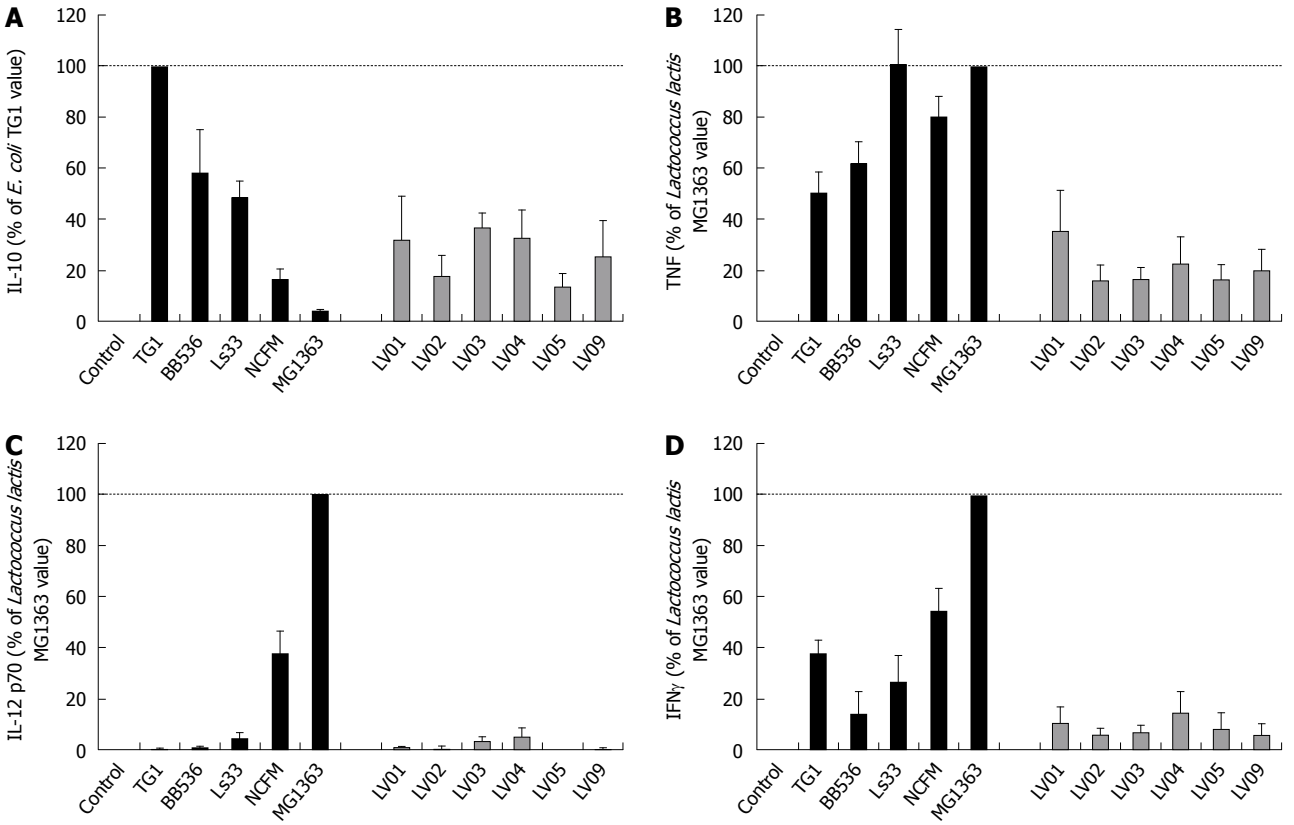


Figure 3 Cytokine production in human PBMCs, stimulated with bacterial reference strains and 6 yeast strains for 24 h. Cytokine levels were analyzed in supernatants by ELISA. A: IL-10; B: TNF; C: IL-12 p70; D: IFNγ. Results are expressed in % of *E. coli* TG1 value for panel A and in % of *Lactococcus lactis* MG1363 for B, C and D, including mean ± SE; *n* = 5.

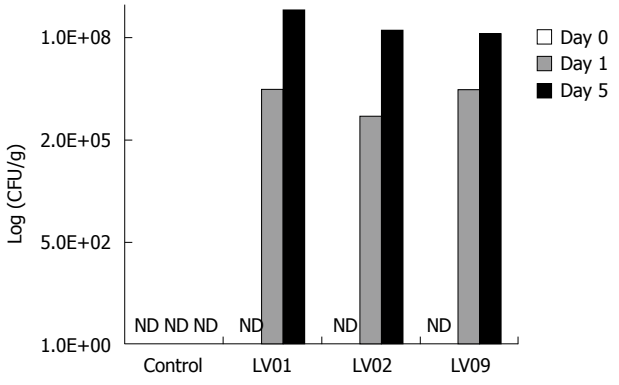


Figure 4 Fecal recovery of the 3 distinct yeast strains in yeast-fed mice, at days 0, 1 and 5. Data were obtained by counting dilutions of stools on appropriate "yeast-selective"-tetracycline agar plates. Results are expressed in Log CFU/g of stools. Results are representative for 2 separate experiments, SD are not represented for clarity reasons. ND: Not detected; CFU: Colony-forming units.

strains also significantly attenuated the deleterious weight changes for the LV01 (-9.9%, $P < 0.01$), and to a lesser extent for LV02 and LV09 (18%, $P < 0.05$). In contrast, the systemic administration of the strain LV01 (LV01-IP) had no additional effect on weight.

After the onset of colitis, anti-inflammatory effects were measured macroscopically for the prednisone-treated as well as for the LV01-treated mice (66% and 27% reduction of macroscopic damage, respectively, $P < 0.01$, Figure 6A). More moderately LV02 reduced the colonic insult (18% of control, $P < 0.05$) while intra-peritoneal injection of LV01 failed to influence the macroscopic scores.

Considering the histological assessment of colitis in various conditions (Figure 6B), both the prophylactic treatment by prednisone and LV01 significantly reduced the microscopic scores of inflammation (by 60.3% and 35%, respectively, $P < 0.05$), while neither LV02 nor LV09 treament could be associated with a significant reduction of the histopathological features observed during active colitis (mucosal erosions, oedema, ulcerations areas and even necrosis). These observations, including thickening of the submucosa and inflammatory infiltrates, were correlated with the colonic myeloperoxidase activities measured (MPO, Figure 6C) and with similar reductions for the steroid drug and LV01 ($P < 0.01$). In contrast, no improvement, and even some aggravation, was depicted for strain LV01 provided by the systemic route, as supported by massive neutrophil infiltrates. Representative aspects of the corresponding colon sections are given (Figure 7). Mouse colons with vehicle-treated TNBS-induced colitis exhibited massive goblet cell depletion and showed disorganized mucosal architecture, including muscle layer necrosis (Figure 7B). By contrast, corticoid-treated animals showed a well-conserved appearance, more similar to normal structures despite a slight thickening of the submucosa. Only minor thickening of submucosa and superficial lesions, mainly

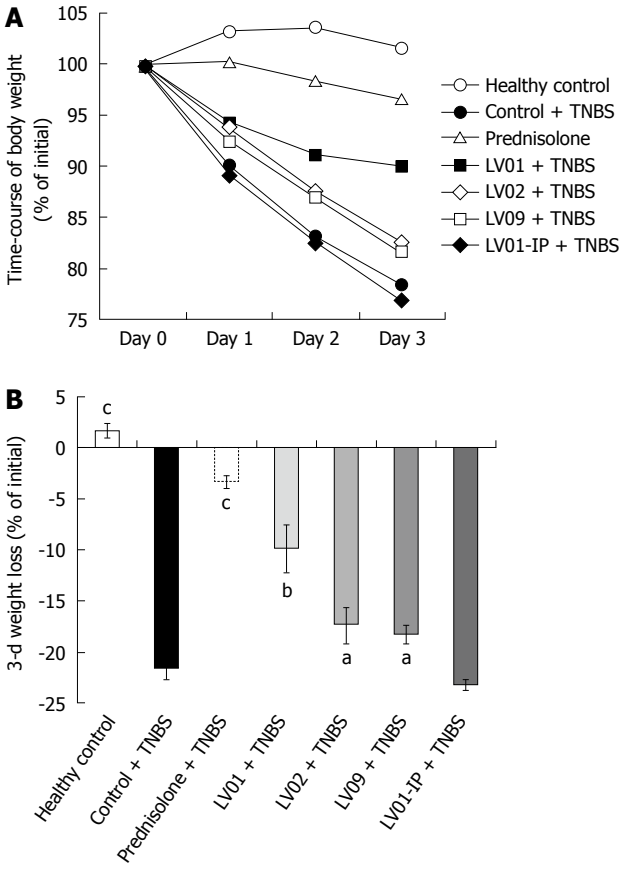


Figure 5 Changes in body weight during the preventive interventional study on experimental colitis. A: Time-course changes of weight for control mice and 2-4-6-trinitrobenzene sulfonic acid (TNBS)-treated mice, covering saline vehicle, prednisone and yeast pre-treated animals. Results are expressed as % of initial weight; SDs are not represented for clarity; B: Final weight loss 3 d after colitis. Results are expressed as mean ± SE (n = 10 per group), $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ *vs* TNBS-saline.

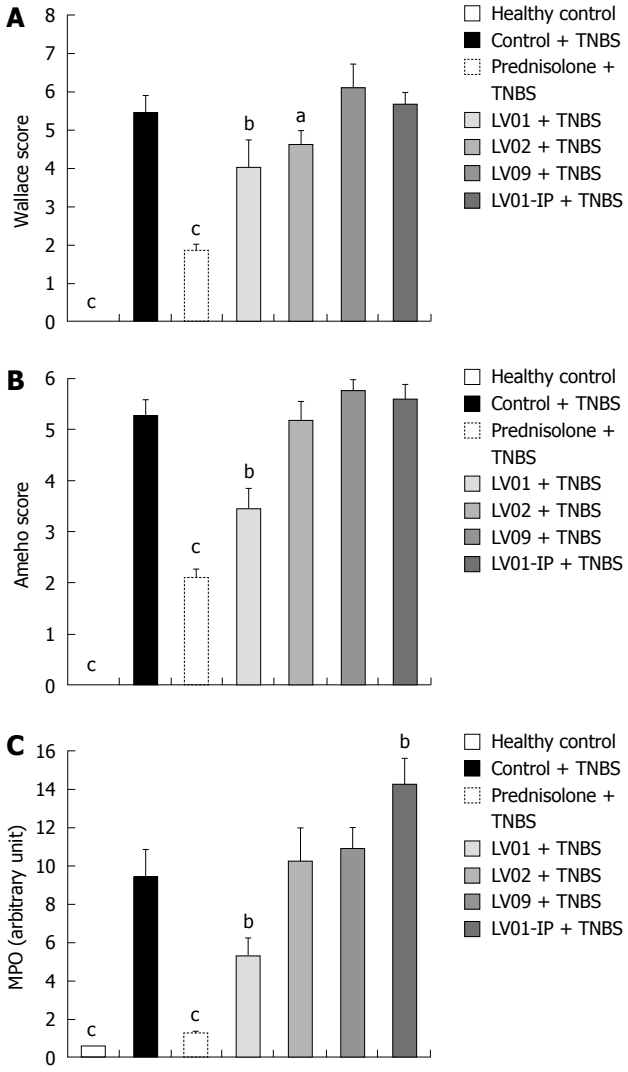


Figure 6 Effects of the various treatments 3 d after the TNBS-induced colitis on colonic macroscopic damage (Wallace) (A), histology scores (Ameho) (B) and myeloperoxidase (MPO) activity (C). Mice received intrarectally either saline or TNBS following oral pre-treatment by prednisone (10 mg/kg, 2 d), by various yeast strains for 5 d or after intra-peritoneal administration (IP) of the yeast at the onset of colitis. Results are expressed as mean ± SE (n = 10 per group), $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ *vs* TNBS-saline.

oedema, were seen in mice treated with the yeast LV01, in complete absence of necrosis. Erosions were more important in LV02-pretreatment, although necrosis was restricted to the upper luminal compartment and the crypt's architecture was still identifiable. Treatment with strain LV09 given orally and strain LV01 after intraperitoneal administration were characterized by prominent inflammatory infiltrates and transmural inflammation with necrosis reaching the deeper muscle layers.

The levels of the blood markers of inflammation SAA and IL-6, while significantly increased after TNBS administration, were strikingly reduced by prednisone and LV01 treatments, approaching baseline values ($P <$ 0.01). LV02 strain also minimized the pro-inflammatory cytokine IL-6 in the serum ($P < 0.05$), with a similar trend observed for SAA ($P = 0.055$, Figure 8).

In conclusion, TNBS caused increases in mucosal and systemic inflammatory symptoms that can be prevented by an orally administered anti-inflammatory drug (prednisone) as well as the food-grade yeast LV01. This was substantiated on the basis of reduction of body-weight loss, macroscopic damage, histological scores, colonic inflammatory infiltrates and serum markers. The yeast strain LV02 showed an intermediate protection capacity with sometimes partial and borderline effects in the degree of attenuation, depending on the marker considered. LV09 only lowered the weight loss with no improvement of other colitis-related symptoms. These results support the relevance of yeast for colitis protection, but distinct performance of various strains highly suggests strain-dependent mechanisms.

DISCUSSION

Evidence that yeasts can be used as a probiotic with therapeutic potential in IBD has emerged from clinical trials and experimental models of colitis in animals. However, these studies remained scarce and essentially used the strain *S. boulardii,* formerly *S. cerevisiae* var *boulardii*. In this study we aimed at questioning the differential anti-inflam-

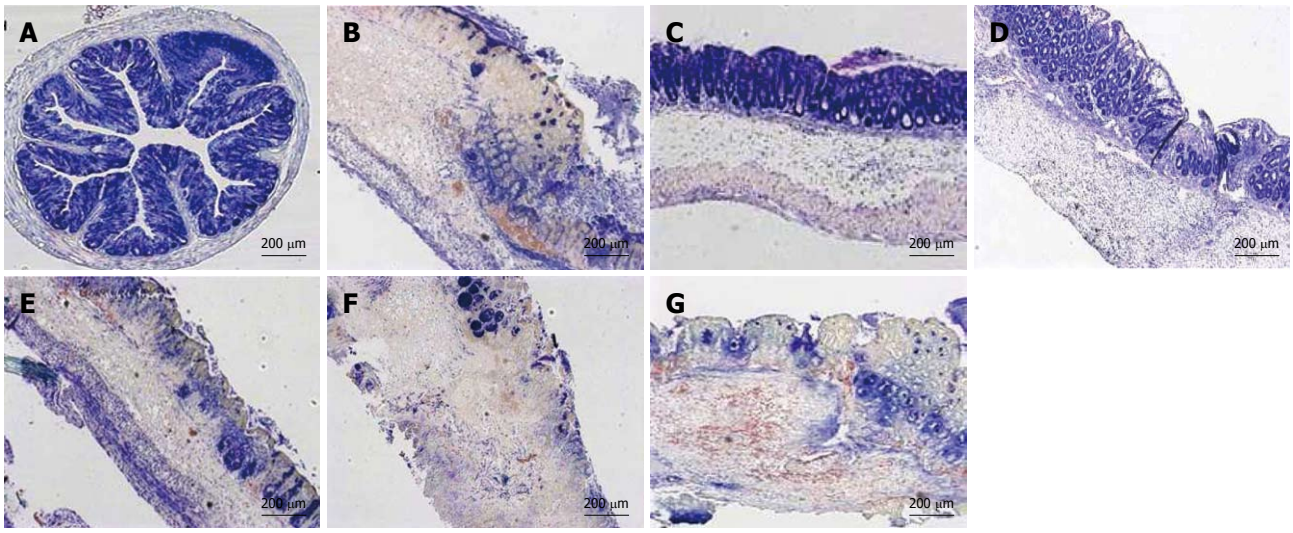


Figure 7 Histological features of yeast-mediated protection. The figure shows representative May-Grünwald and Giemsa-strained colon sections of control healthy mice (A) or day 3 after instillation of TNBS in Balb/c mice pre-treated with either vehicle (B), prednisone (C), LV01 (D), LV02 (E), LV09 (F) and LV01-IP (G). Original magnification, × 20.

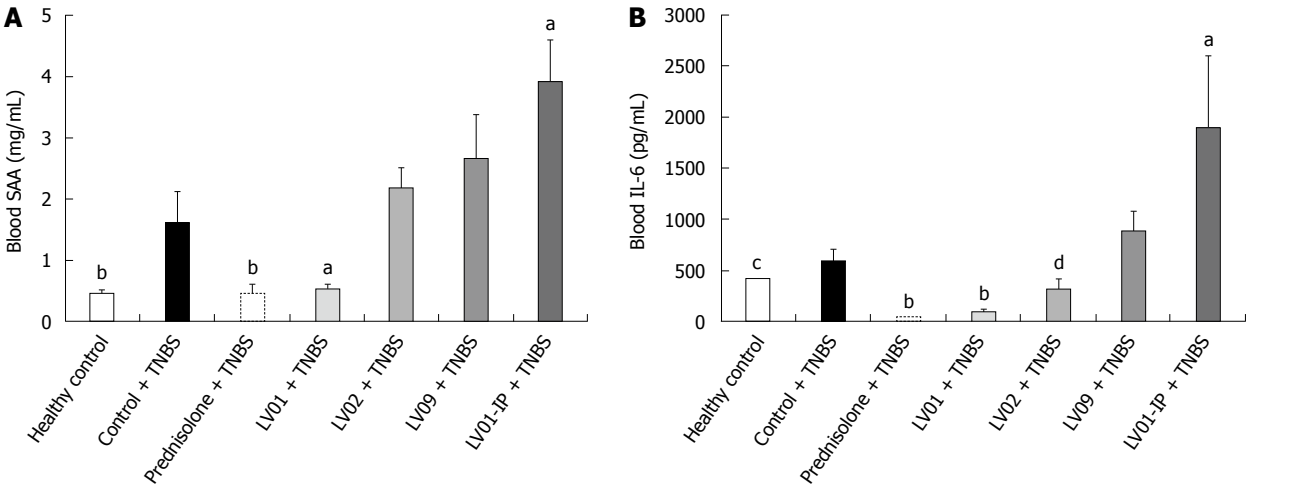


Figure 8 Effects of the various treatments 3 d after the TNBS-induced colitis on blood markers. A: Serum amyloid A protein (SAA); B: IL-6. Mice received intra-rectally either saline or TNBS following oral pre-treatment by prednisone (10 mg/kg, 2 d), by various yeast strains for 5 d or after intra-peritoneal administration (IP) of the yeast at the onset of colitis. Results are expressed as mean ± SE (n = 10 per group), $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$, $0.05 < {}^{d}P < 0.1$ *vs* TNBS-saline.

matory potential of other yeast strains. Whether food-borne strains of *S. cerevisiae* or other yeast species possess probiotic properties is of fundamental importance in developing, understanding and handling of new potential biotherapeutic agents, both concerning the pharmaceutical and the (functional) food industry. Attempts for such a screening of yeast biodiversity were already carried out *in vitro*[17,35,36], mainly by studying selection criteria such survival at low acid pH and tolerance to bile salts or adhesion on epithelial cells. The predictability for the *in vivo* situation, however, is limited, as the importance of colonization in strain efficacy remains to be determined *in vivo*[37]. An example was recently given with a *Lactobacillus* strain that fulfilled adhesion and survival criteria *in vitro* but was unable to persist in mice, although exerting immunomodulatory effects[38]. To our knowledge, the comparison of distinct yeast strains based on their ability to induce cytokine secretions by immunocompetent cells was never reported.

Results obtained in this study showed that, in contrast to bacteria[22,39,40], *in vitro* immunomodulation did not discriminate among various strains. This can be attributed to the more conserved cell-wall structure of the yeasts. However, yeast cells are not inert from an immunological point of view and substantial induction of IL-10, on PBMCs, associated with weak TNF and no release of IFNγ and IL-12, favor, in general, a more anti-inflammatory profile than bacteria. This is in agreement with very recent studies using germ-free mice[41].

The three strains used in the murine TNBS-induced colitis exhibited similar colonization patterns *in vivo*, thus allowing accurate comparisons. Standardization of experimental settings for all evaluations and, as far as possible into the same study, is highly recommended[42]. The

present work confirms that a yeast strain (*S. boulardii*) is able to significantly prevent symptoms in an acute experimental colitis in mice. The 30% protection observed, lower than the protection obtained by a traditional anti-inflammatory drug, is consistent with previous reports using the same yeast strain in a rat model of TNBS[13]. By contrast, in another study in rats, TNBS-induced inflammation was only weakly lowered with *S. boulardii* with the 18% trend, which did not passed the statistics test, while conventional IBD drugs and other probiotic preparations did show significance[43,44]. Such differences between studies may depend on experimental design including distinct outcomes when considering preventive *vs* therapeutic applications, as reported e.g. for bacteria in the TNBS colitis model[45], in the experimental DSS model[46] or even in clinical studies[47]. Hence, these observations emphasize the necessity to compare distinct treatments and strains in a single study. We enlightened here for the first time that, like probiotic bacteria, probiotic yeasts are not equivalent in their capacity to alleviate colitis, despite a similar colonization profile.

The "main contribution" of yeast-mediated anti-inflammatory effects is suggested to more address the intestinal barrier properties and epithelial cells rather than an immunocompetent cell-based immunity, because (1) protection triggered by yeasts *in vivo* is strain-dependent, whereas together with the immune screening *in vitro*, was poorly discriminating; and (2) the failure of *S. boulardii* to induce protection by the systemic route, in contrast to the oral route. Indeed, in contrast to some beneficial bacteria which are able to exert effects distantly from the inflammation site[33,34,48], emphasizing a possible role of migratory cells such as dendritic cells (DC)[31], yeast-mediated protection seems to take place predominantly in the intestinal mucosa. An intestinal barrier dysfunction in IBD is obvious, although it is not clear yet whether this is a cause or a consequence of the disease[49]. Prophylactic reinforcement and therapeutic restoration of barrier function by changing the luminal environment may successfully stimulate the mucosal barrier. Probiotics are elements of choice to normalize the barrier function, stabilize tight junction components[50] and limit translocation[51]. Trophic effects[52] as well as enhancement of epithelial integrity mediated by yeasts have been extensively described[4,7]. Reduction of bacterial translocation by oral treatment with yeasts in various models of sepsis also confirmed this functional aspect of probiotic-yeasts[53-55].

However, other mechanisms including direct yeast-DC interactions were also evoked to explain how *S. boulardii* exhibits its anti-inflammatory properties[56]. Stimulation may occur in Peyer's patches or in the lamina propria. This mechanism did not seem to be very strain specific and, paradoxically, even *Candida albicans* was recently reported to induce tolerogenic DC and to positively balance inflammation after adoptive transfer in a mice colitis model[57]. Similarly, soluble β-glucans derived from *C. albicans* were able to suppress the production of pro-inflammatory cytokines by PBMCs[58] while mannan from *S. cerevisiae* impaired the ability of macrophages to kill phagocytosed pathogens[59]. On the other hand, colonization of mice by *C. albicans* exacerbated DSS-colitis[60] and delayed healing of TNBS colitis[61]. Finally, intraperitoneal injection of zymosan, a polysaccharide cell wall component from *S. cerevisiae* has been widely used as a (self-resolving) model of acute inflammation[62]. By contrast, yeast extracts administered to mice *per os* resulted in a significant reduction of the *C. difficile*-toxin A-mediated COX-2 increase and in the toxin-mediated disruption of epithelial cells[63]. Whether yeast or yeast-derived molecules will have a pro- or anti-inflammatory role may thus greatly depend on the route of administration and consequently on the target cells and tissues.

Nevertheless, we can speculate that oral yeast-induced immunomodulation predominantly addresses intestinal permeability and, based on published data, the immune function of intestinal cells. This is consistent with clinical findings indicating that *S. boulardii* has a positive effect on the maturation of enterocytes, but only a minor influence on lymphocytes[64]. Our results suggest that further screening for probiotic yeasts may favor the use of epithelial cell or mucosa-integrated models, rather than immune-cell based approaches. This will help us to select yeast or fungal strains with important potential in inflammatory conditions or in a context where inflammation is concomitant with enteropathogens such *C. difficile* and *E. coli*[65]. Understanding how various strains perform *in vivo* will allow us to trace specific mechanisms and select or design optimal strains for specific infectious or inflammatory diseases, or even consider new probiotic applications[66]. As some of the yeast-based probiotic mechanisms seem to be distinct from the bacterial ones, possible synergy might be expected from mixing both types of probiotics[67]. This was previously shown in a preclinical study on traveler's diarrhea, where the combination of yeast and bacteria was found to be more effective than each probiotic alone[68]. Using probiotic *Saccharomyces spp.* alone or in combination with lactic acid bacteria will thus enhance and extend the actual biotherapeutic/probiotic approach in IBD together with conventional anti-inflammatory treatments. Lastly, the strain-specific differences seen in the anti-inflammatory capacity of yeasts may also be extended for clinical use against *Salmonella ssp.*, *C. difficile* and *Candida species*, requiring an optimal and controlled formulation of the probiotic product[69].

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COMMENTS

Background

As for probiotic bacteria, probiotic yeast such *Saccharomyces boulardii*

(*S. boulardii*) can be effective in gastrointestinal pathologies such as inflammatory bowel disease (IBD) and bacterial- or enterotoxin-mediated diarrhea and inflammation. However, very few yeast strains have been studied as possible biotherapeutics agents. The underlying mechanisms of action of probiotics are not fully understood but there is some evidence that they exert their beneficial effect by multiple and specific modes of action depending on the type of the probiotic (bacteria, yeast), the species and strain.

Innovations and breakthroughs

The yeast-mediated capacity to lower inflammation is suggested to mainly involve epithelial cells and barrier function, as demonstrated by (1) the weakness of specific immunomodulatory response *in vitro*; and (2) the lack of protection by *S. boulardii* when administered by the systemic but not the oral route. In contrast with the performance of some probiotic bacteria which the authors previously showed to be directly correlated with their *in vitro* anti-inflammatory potential, the immunoregulatory mechanism of probiotic yeasts fails to directly act on the cytokine level.

Applications

As for probiotic bacteria, strain specificity also matters for yeast as for probiotics, but mechanisms, and consequently screening methods, partly differ between yeast and bacteria. A careful and appropriate selection of strains is then required to screen the biodiversity of yeasts for specific clinical studies including applications in IBD as well as for other dedicated therapeutic uses. This will impact on future product composition to enhance or synergize gastrointestinal related disorders by using probiotics.

Terminology

Probiotics are defined as living microorganisms that may have beneficial effects on health, including mainly food-grade bacteria and non-pathogenic yeasts.

Peer review

The paper by Benoit Foligné and co-workers investigated the anti-inflammatory effects of various yeasts strains in experimental colitis in mice. The overall design of the study is fair and the results are stimulating for future research.

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BRIEF ARTICLE

Prophylaxis for venous thromboembolism after resection of hepatocellular carcinoma on cirrhosis: Is it necessary?

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Abstract

AIM: To assess the safety and effectiveness of prophylaxis for venous thromboembolism (VTE) in a large population of patients with hepatocellular carcinoma (HCC) on cirrhosis.

METHODS: Two hundred and twenty nine consecutive cirrhotic patients with HCC who underwent hepatic resection were retrospectively evaluated to assess whether there was any difference in the incidence of thrombotic or hemorrhagic complications between those who received and those who did not receive prophylaxis with low-molecular weight heparin. Differences and possible effects of the following parameters were investigated: age, sex, Child-Pugh and model for end-stage liver disease (MELD) score, platelet count, presence of esophageal varices, type of hepatic resection, duration of surgery, intraoperative transfusion of blood and fresh frozen plasma (FFP), body mass index, diabetes and previous cardiovascular disease.

RESULTS: One hundred and fifty seven of 229 (68.5%) patients received antithromboembolic prophylaxis (group A) while the remaining 72 (31.5%) patients did not (group B). Patients in group B had higher Child-Pugh and MELD scores, lower platelet counts, a higher prevalence of esophageal varices and higher requirements for intraoperative transfusion of FFP. The incidence of VTE and postoperative hemorrhage was 0.63% and 3.18% in group A and 1.38% and 1.38% in group B, respectively; these differences were not significant. None of the variables analyzed including prophylaxis proved to be risk factors for VTE, and only the presence of esophageal varices was associated with an increased risk of bleeding.

CONCLUSION: Prophylaxis is safe in cirrhotic patients without esophageal varices; the real need for prophylaxis should be better assessed.

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Key words: Hepatic surgery; Hepatocellular carcinoma; Liver cirrhosis; Postoperative bleeding; Postoperative thromboembolism; Venous thromboembolism prophylaxis

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INTRODUCTION

Venous thromboembolism (VTE) is a significant cause of morbidity and mortality in patients who have undergone open gastrointestinal surgery, particularly if they were operated on due to malignancy[1,2].

Hepatocellular carcinoma (HCC) is the most frequent primary neoplasm of the liver and often develops as a consequence of chronic liver disease[3]. It is well known that several hemostatic alterations are present in patients with liver disease: primary hemostasis is often impaired due to piastrinopenia and secondary hemostasis can be hampered by the reduced synthesis of coagulation factors that normally takes place in the liver[4]. These alterations can be worsened by the decrease in hepatic volume caused by hepatic resection. A historical series of cirrhotic patients undergoing liver resection reported an 8.4% incidence of hemorrhagic complications following surgery[5].

While there is currently agreement on the need for pharmacological prophylaxis of VTE in surgical patients in general, little is know about the effect of this prophylaxis in patients with chronic liver disease who undergo hepatic resection due to the presence of HCC.

Studies on the coagulative pattern have shown that in patients with chronic liver disease there is decreased production of natural anticoagulant proteins that could result in an increase in the risk of thrombotic events; however, in clinical studies the incidence of VTE in cirrhotic patients seems to be lower than in the general medicine population[6].

Although great advances have taken place in the field of hepatobiliary surgery, resection of a cirrhotic liver remains a challenging procedure and hepatic surgeons may not feel confident in administering antithromboembolic prophylaxis to patients who have an increased risk of bleeding.

In our centre, where a large number of hepatic resections are performed, we have more and more frequently adopted a scheme of prophylaxis with low-molecular-weight heparin, however, we have no clear-cut parameters to refer to when deciding whether to administer prophylaxis or not and a significant number of patients are not given prophylaxis due to the risk of bleeding.

The aim of the present study was to assess the possible effect of different prophylactic strategies in the prevention of venous thrombosis in a large series of cirrhotic patients who underwent hepatic resection for HCC.

MATERIALS AND METHODS

The records of 229 consecutive patients with chronic liver disease who underwent hepatic resection due to the presence of HCC between January 1999 and December 2008 were retrospectively reviewed to ascertain whether there was any difference in the incidence of venous thromboembolic or hemorrhagic complications between those patients who received venous thrombosis prophylaxis and those who did not.

Table 1 Baseline characteristics of the study population (mean ± SD) *n* (%)

Variables	All patients (*n* = 229)	Group A (*n* = 157)	Group B (*n* = 72)	*P* value
Age (yr)	65.0 ± 9.3	65.0 ± 9.8	63.0 ± 9.5	0.080
Gender (M/F)	171 (74.7)/ 58 (25.3)	119 (76.0)/ 38 (24.0)	52 (72.0)/ 20 (28.0)	0.330
Etiology of cirrhosis				0.200
Hepatitis C	148 (64.6)	106 (67.0)	42 (58.3)	
Hepatitis B	37 (16.2)	21 (14.0)	16 (22.2)	
Hepatitis B + C	15 (6.6)	7 (5.0)	8 (11.1)	
Non-viral	29 (12.7)	23 (14.0)	6 (8.3)	
Child-Pugh score				0.005
A	218 (95.2)	154 (98.0)	64 (89.0)	
B	11 (4.8)	3 (2.0)	8 (11.0)	
C	0			
MELD score	8.8 ± 1.5	8.5 ± 1.2	9.3 ± 1.9	0.001
Platelet count (× 10^3)	139 ± 60	150 ± 60	115 ± 51	0.001
Esophageal varices	63 (28.0)	34 (22.0)	29 (40.3)	0.003

MELD: Model for end-stage liver disease.

All patients in the present series had the diagnosis of chronic liver disease confirmed by histology which was carried out on the resected specimen. Etiology of liver disease was predominantly viral (87.3% of cases) (Table 1).

Preoperative Child-Pugh-Turcotte score[7] and model for end-stage liver disease (MELD)[8] score were available for all patients; other parameters that were taken into account to determine the severity of portal hypertension were platelet count and presence of esophageal varices. Two hundred and eighteen patients (95.2%) had class A liver disease according to the Child-Pugh scoring system, while 11 (4.8%) had class B and none had class C disease; the mean MELD score of the patients was 8.8 ± 1.5.

Body mass index (BMI), diabetes mellitus and clinical history of cardiovascular disease were recorded as possible risk factors for thromboembolism. Type of surgical resection (minor when one or less and major when more than one hepatic segment was resected), duration of surgery, intraoperative transfusion of blood and fresh frozen plasma (FFP) were also recorded (Tables 2 and 3). Minor hepatic resection was carried out in 219 cases (95%), while major hepatic resection was performed in 10 cases (5%).

A protocol for general surgical procedures for the prevention of venous thrombosis using low-molecular-weight heparin prophylaxis was always observed in the period considered here. In the case of hepatic resection in cirrhotic patients the decision on whether to use prophylaxis was left to the judgment of the surgeon who performed the resection based on preoperative coagulation tests and intraoperative findings. For those patients

Table 2 Details of surgery performed in the two groups

Variables	All patients (*n* = 229)	Group A (*n* = 157)	Group B (*n* = 72)	*P* value
Type of resection *n* (%)				0.610
Minor	219 (95.0)	150 (95.5)	69 (95.8)	
Major	10 (5.0)	7 (4.5)	3 (4.2)	
Duration of surgery (mean ± SD)	266 ± 99	261 ± 106	275 ± 83	0.320
i.o. blood transfusion [mL, median (range)]	128.2 (0-1500)	124.7 (0-1500)	154.1 (0-1200)	0.346
i.o. FFP transfusion [mL, median (range)]	175.7 (0-1200)	139.5 (0-1200)	266.6 (0-1200)	0.003

i.o.: Intraoperative; FFP: Fresh frozen plasma.

who were administered anticoagulant prophylaxis, this consisted of nadroparin calcium 0.3 mL or enoxaparin sodium 0.4 mL subcutaneously, starting from the day of surgery and continued for at least 7 d or until the patient was actively ambulant.

Prophylaxis was stopped when hemorrhagic complications developed.

Independent of the use of low-molecular-weight heparin, all patients had mechanical prophylaxis by means of anti-embolism stockings.

Patient follow-up included evaluation at the outpatient clinic on days 7 and 30 and months 3, 6, 9 and 12 after discharge from the Hospital.

VTE was defined as the symptomatic or asymptomatic occurrence of deep vein thrombosis confirmed by Doppler ultrasonography or venography; pulmonary embolism was confirmed by helical computed tomography.

Hemorrhagic complications were defined as follows: (1) Blood loss from surgical drainage associated with a significant drop in hemoglobin levels (> 1.5 g/dL from the last control); (2) Intraabdominal fluid collection with density at CT compatible with blood of diameter > 3 cm; and (3) Bleeding from the upper or lower gastrointestinal tract.

The analysis of risk factors for VTE included the following parameters: (1) age; (2) sex; (3) etiology of chronic liver disease; (4) Child-Pugh score; (5) MELD score; (6) presence of esophageal varices; (7) platelet count; (8) BMI; (9) chronic heart disease; (10) diabetes; (11) extent of surgical resection (major when more than 1 hepatic segment was resected); (12) duration of surgery; (13) intraoperative requirement for blood or FFP transfusion; and (14) prophylaxis with low-molecular weight heparin.

Statistical analysis

Continuous variables are reported as mean ± SD or in median and range on the basis of parametric assumption; differences between subgroups were investigated with Levene's test for equality of variances and compared with the Student *t*-test or Mann-Whitney test as appropriate. Categorical variables were reported in a number of cases and prevalence and differences in subgroups were compared using the χ^2 test with Yates correction. Univariate logistic regression analysis was applied in order to investigate risk factors for thrombosis or hemorrhage. *P*-values less than 0.05 were considered statistically significant in all the analyses. Statistical analysis was performed using the SPSS for Windows package (Version 10.0).

Table 3 Specific risk factors for venous thromboembolism in the two groups *n* (%)

Variables	All patients (*n* = 229)	Group A (*n* = 157)	Group B (*n* = 72)	*P* value
BMI > 30 kg/m^2	36 (17.1)	26 (17.8)	10 (15.4)	0.410
Diabetes	47 (20.5)	33 (21.0)	14 (19.4)	0.460
Cardiovascular disease	56 (24.5)	43 (27.0)	13 (18.1)	0.080

BMI: Body mass index.

RESULTS

One hundred and fifty-seven of the 229 (68.5%) patients received antithromboembolic prophylaxis (Group A) while the remaining 72 (31.5%) patients did not (Group B). The proportion of patients who received prophylaxis significantly varied with time: prophylaxis was given to 48 of 99 (48.4%) patients in the period 1999-2003 and to 109 of 130 (83.8%) patients in the period 2004-2008 (*P* = 0.001).

There was no difference in age, gender and etiology of liver disease between the 2 groups, while patients in group B had higher Child-Pugh and MELD scores, lower platelet counts and a higher prevalence of esophageal varices (Table 1).

Extent and duration of the hepatic resection and intraoperative blood transfusions were similar in the 2 groups, while in group B there was a significantly higher requirement for intraoperative transfusion of FFP (Table 2). This latter finding might indicate a higher bleeding tendency observed by the surgeon in the operative field, which resulted in the administration of greater amounts of FFP.

As regards the specific risk factors for venous embolism that were considered, there was no difference between the 2 groups (Table 3).

Two cases (0.87%) of deep venous thrombosis were observed: these were one case of pulmonary embolism secondary to a deep vein thrombosis of the leg in a patient who was receiving prophylaxis and one case of total portal vein thrombosis in a patient who did not receive prophylaxis: this latter patient had a Child score of A6, a MELD score of 13 with a low platelet count (113.000/mL) an INR = 1.3 and no esophageal varices, and underwent wedge resection and died of hepatic failure. The incidence of VTE was therefore 1.38% in patients who did not receive prophylaxis and 0.63% in those patients who were treated with low-molecular-weight heparin (*P* = 0.530).

Six cases of hemorrhagic complications were observed (2.62%); 5 of these cases occurred in those 157

Table 4 Analysis of possible risk factors for venous thromboembolism

Variable	Exp (B)	95% CI	*P* value
Child-Pugh score (score A *vs* B)	0.004	0.00-15.32	0.906
MELD score	1.748	0.96-3.16	0.132
Type of resection (minor *vs* major)	0.004	0.00-17.52	0.914
Platelet count	0.990	0.99-1.00	0.776
Esophageal varices	0.001	0.00-18.22	0.525
BMI	0.001	0.00-11.45	0.687
Diabetes	0.001	0.00-12.45	0.631
Cardiovascular disease	0.001	0.00-19.31	0.570
Duration of surgery	0.997	0.98-1.01	0.720
i.o. blood transfusion (mL)	1.000	0.99-1.00	0.490
i.o. FFP transfusion (mL)	1.001	0.99-1.00	0.518
VTE prophylaxis	0.455	0.02-7.38	0.531

Table 5 Analysis of possible risk factors for hemorrhagic complications

Variable	Exp (B)	95% CI	*P* value
Child-Pugh score (score A *vs* B)	0.004	0.00-23.72	0.742
MELD score	1.280	0.83-1.95	0.252
Type of resection (minor *vs* major)	0.004	0.00-20.75	0.763
Platelet count	0.987	0.99-1.02	0.445
Esophageal varices	5.559	0.99-31.14	0.050
BMI	0.000	0.00-27.45	0.389
Diabetes	0.000	0.00-24.28	0.248
Cardiovascular disease	1.564	0.27-8.77	0.454
Duration of surgery	0.997	0.98-1.00	0.460
i.o. blood transfusion (mL)	1.001	0.99-1.00	0.315
i.o. FFP transfusion (mL)	1.002	0.98-1.00	0.073
VTE prophylaxis	0.443	0.26-20.42	0.387

VTE: Venous thromboembolism.

patients who received prophylaxis (prevalence: 3.18%) and one in patients who did not receive prophylaxis (prevalence: 1.38%); the difference was not significant ($P = 0.380$). The hemorrhagic complications consisted of prolonged blood leakage from the surgical drains requiring blood transfusion (3 cases), intraperitoneal blood collection (2 cases) and gastric bleeding (1 case). Only 1 of the above-mentioned cases required invasive intervention (CT-guided percutaneous drainage of intraperitoneal collection).

None of the considered risk factors proved to be significantly associated with VTE in the univariate analysis, and only the presence of esophageal varices was linked to an increased risk of hemorrhagic complications (Tables 4 and 5).

DISCUSSION

Despite the clinical relevance of this topic, to our knowledge no reports have been published to date on the use of venous thrombosis prophylaxis in cirrhotic patients undergoing hepatic resection for HCC. The absence of clear guidelines for prophylaxis of thromboembolism in the specific setting of hepatic surgery in patients such as those with chronic liver disease who are known to have imbalances in coagulative function may induce those surgeons with less experience in hepatobiliary surgery to avoid prophylaxis for fear of hemorrhagic complications; on the other hand, administering prophylaxis to patients with severe coagulative impairment and severe portal hypertension possibly following an extensive liver resection should be done on more solid grounds than simple adherence to existing guidelines that were drawn up for open abdominal surgery. In both cases, should thromboembolic or hemorrhagic complications develop, then the possibility of a malpractice accusation would be consistent[9].

In our experience, we found a particularly low incidence of deep vein thrombosis in cirrhotic patients who underwent liver resection, which could be partially justified by the absence of specific postoperative screening; however, at our center, HCC patients are always closely monitored with follow-up after resection and we can therefore exclude the fact that major thrombotic complications took place after hospital discharge which do not show up in the clinical records. Of note, all the patients in this report received prophylaxis with a mechanical method (anti-embolism stockings) which, together with the peculiarities of the study population, may have played a role in keeping the rate of VTE particularly low.

Hemorrhagic complications after liver resection are often poorly defined in specific reports, and the impression is that only those that require invasive maneuvers are usually reported; nevertheless, the incidence of these complications after resection in cirrhotic patients ranges between 1% and 8%[5,10,11]. The hemorrhagic complications reported in the present study were mainly represented by prolonged blood leakage from the surgical drains requiring blood transfusion. Although a trend towards a higher incidence of hemorrhage following resection was observed in those patients who received prophylaxis, the difference between the 2 groups was not significant.

Prophylaxis was withheld mainly in those patients who had a higher Child-Pugh or MELD score, lower platelet count and higher prevalence of esophageal varices; however, the overall increase in prophylaxis administration over time reflects an increase in confidence in handling it.

The surgeon performing the hepatic resection can assess some elements such as the bleeding tendency observed in the operative field and the degree of portal hypertension as revealed by the number and size of hepatofugal collateral veins, which can provide information that goes beyond what can be assessed with laboratory and imaging screening. However, the categorization of these elements largely depends on the degree of experience and confidence of the surgeon, and their effective relationship with the risk of bleeding in the postoperative period is difficult to demonstrate.

Similarly, in the cirrhotic patient, the common coagulative tests such as prothrombin time or INR poorly predict the effective risk of bleeding: in fact, these tests

only measure the activity of procoagulant factors while they ignore possible changes in the activity of anticoagulant factors that, in liver disease, are also deficient[12].

In our study, a greater amount of FFP was administered to those patients who did not receive prophylaxis, which might reflect a bleeding tendency that was observed at surgery. We believe that, unless clear guidelines are validated by large clinical trials, antithromboembolic prophylaxis should probably be avoided in those patients who are judged at high risk of bleeding by the surgeon.

Despite their low incidence in this series, the thromboembolic complications seen after surgery were life-threatening, however, we did not experience similar serious hemorrhagic complications. In the absence of more reliable tools to quantify the probability of bleeding it seems reasonable to avoid prophylaxis when portal hypertension is present or significant bleeding is observed at surgery.

Further prospective studies are necessary to establish guidelines for application in this specific setting.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is the most common primary neoplasm of the liver and often arises in the context of a chronic liver disease that impairs coagulative function. Surgical resection is the best option to cure HCC, however, surgery on cirrhotic liver may increase the risk of bleeding. Despite the clinical relevance of the matter, no guidelines are available on the administration of antithromboembolic prophylaxis in cirrhotic patients undergoing hepatic resection.

Innovations and breakthroughs

The study shows that the risk of venous thromboembolism after hepatic resection in cirrhotic patients is low. A trend towards an increased incidence of postoperative hemorrhage was observed in patients who received prophylaxis. Portal hypertension, as demonstrated by the presence of esophageal varices, is significantly associated with the risk of bleeding.

Applications

The absence of guidelines for prophylaxis of thromboembolism specifically aimed at patients with chronic liver disease undergoing hepatic surgery can lead to the generalized application of prophylactic schemes which are used in the setting of open abdominal surgery, thus increasing the risk of bleeding. Conversely, to withold prophylaxis might increase the risk of thromboembolism. Both these policies can be dangerous and lead to malpractise accusations. Prospective studies are necessary to establish specific guidelines.

Peer review

This is a good manuscript to get published but needs some more information. The manuscript is well written and would be helpful in the field of hepatic surgery.

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BRIEF ARTICLE

Impact of bolus volume on small intestinal intra-luminal impedance in healthy subjects

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Abstract

AIM: To assess the impact of bolus volume on the characteristics of small intestinal (SI) impedance signals.

METHODS: Concurrent SI manometry-impedance measurements were performed on 12 healthy volunteers to assess the pattern of proximal jejunal fluid bolus movement over a 14 cm-segment. Each subject was given 34 boluses of normal saline (volume from 1 to 30 mL) *via* the feeding tube placed immediately above the proximal margin of the studied segment. A bolus-induced impedance event occurred if there was > 12% impedance drop from baseline, over ⩾ 3 consecutive segments within 10 s of bolus injection. A minor or major impedance event was defined as a duration of impedance drop < 60 s or ⩾ 60 s, respectively.

RESULTS: The minimum volume required for a detectable SI impedance event was 2 mL. A direct linear relationship between the SI bolus volume and the occurrence of impedance events was noted until SI bolus volume reached 10 mL, a volume which always produced an impedance flow event. There was a moderate correlation between the bolus volume and the duration of impedance drop (r = 0.63, P < 0.0001) and the number of propagated channels (r = 0.50, P < 0.0001). High volume boluses were associated with more major impedance events (⩾ 10 mL boluses = 63%, 3 mL boluses = 17%, and < 3 mL boluses = 0%, P = 0.02).

CONCLUSION: Bolus volume had an impact on the type and length of propagation of SI impedance events and a threshold of 2 mL is required to produce an event.

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Key words: Bolus volume; Health; Impedance; Luminal flow; Small intestine

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INTRODUCTION

Absorption of nutrients is the main function of the

small intestine and requires not only normal mucosal integrity but also normal motor function[1-4]. Adequate mixing and proper transit of chyme are important in ensuring optimal absorption[3,4]. Accurate assessment of small bowel motor function is important for understanding the physiology of the gastrointestinal tract, as well as assessment of clinical disorders of the intestine[1,5-7]. Currently, several tests are available for the assessment of intestinal motility and transit. Although intra-luminal manometry detects contractile patterns, it provides only indirect data regarding flow[5]. Similarly, although intestinal transit can be measured by conventional scintigraphy and more recently by breath tests, these methods detect only total transit time and not patterns of flow; transit can be affected by gastric emptying rate and conditions such as bacterial overgrowth[5]. Fluoroscopic studies, on the other hand, are able to assess overall intestinal motility and bolus transit, but are greatly limited by the exposure of patients to radiation[5].

In combination with manometry, multiple intra-luminal impedance (MII) has been introduced as a technique to assess motility and bolus transit concurrently[8-10]. Although this combined technique has been validated and applied extensively in the study of oesophageal physiology and disease[11-14], few data are available regarding its use in the evaluation of small bowel motor function[15-17]. Recently, detailed analysis from a study using combined video-fluoroscopy, manometry and MII showed that an impedance drop of > 12% from the baseline which propagated over 5 cm is associated with a flow event as seen on fluoroscopy[17]. Furthermore, various patterns of impedance flow events during fasting and in the postprandial state have been reported in the proximal intestine of healthy subjects[16]. This study, however, did not have concurrent manometric assessment and it is unclear whether the variation in impedance patterns of flow was influenced by intestinal motor activity or volume of the transported bolus or chyme. Knowledge on the relationship between bolus volume and impedance changes in the oesophagus[18] allows investigators to use intra-luminal impedance to assess oesophageal flow and clearance, without the need for radiological assessment. Corresponding data for the small intestine, however, are lacking. The aim of the current study was to assess the impact of bolus volume on the characteristics of small intestinal (SI) impedance signals in healthy humans.

MATERIALS AND METHODS

Subjects

Studies were performed in 12 healthy subjects (6 males; age: 53 ± 6 years; body mass index: 24.5 ± 1.1 kg/m^2) at the Department of Gastroenterology and Hepatology, Royal Adelaide Hospital. Exclusion criteria included previous or current gastrointestinal symptoms or surgery, evidence of acute or chronic illness and medications known to influence gastrointestinal motor function. The protocol was approved by the Research Ethics Committee of the Royal Adelaide Hospital and all subjects gave written informed consent.

SI manometry and impedance recording

SI motility and intraluminal electrical impedance were recorded concurrently using (1) a 110-cm perfused multilumen manometric assembly (DentSleeve Pty Ltd., Wayville, Australia); and (2) an impedance catheter (Sandhill Scientific, Highland Ranch, CO, USA).

Intestinal motility was recorded using an assembly comprising 11 pressure recording side holes: eight spaced at 2 cm intervals and the following three at 10 cm intervals, from the catheter tip. All manometric lumina were perfused with degassed distilled water at a rate of 0.15 mL/min, by a pneumo-hydraulic capillary perfusion pump (Dentsleeve).

SI luminal flow was recorded using an electrode with eight impedance rings (4 mm in length) spaced 2 cm apart, enabling 7 consecutive recording segments over a distance of 14 cm (Z1 to Z7; proximal to distal, respectively). Each segment straddled a corresponding manometric side hole and was activated by a high frequency (1 kHz) low amplitude (< 6 μA) alternating current.

Manometric and impedance signals were recorded simultaneously using a specialised computer system (Insight Acquisition, Sandhill Scientific) and displayed and stored on a personal computer for subsequent display and analysis.

Duodenal feeding tube

An 18F feeding tube was used to deliver the liquid boluses. The tube was attached to the manometry-impedance assembly and was positioned so that its tip was 1-cm above the most proximal impedance recording segment, Z1. The assembly configuration is outlined in Figure 1.

Protocol

All subjects were studied after an overnight fast. The assembly was passed into the stomach to a distance of 65 cm, through an anaesthetized nostril. The assembly was then allowed to migrate naturally into the duodenum *via* peristalsis, which was monitored continuously by measurements of the antro-duodenal trans-mucosal potential difference (TMPD). The final catheter position was achieved when the most proximal manometric side-hole was located in the duodenum, confirmed by a TMPD reading ⩾ -15 mV[19]. In this position, the combined manometry-impedance recording segment would be expected to lie in the proximal jejunum and at least 20 cm distal to the pylorus. Radiological confirmation of the catheter position was not performed due to the concern of unnecessary radiation exposure to healthy subjects.

Once the assembly was positioned in the proximal jejunum, subjects were positioned in a 30 degree head-up, supine position. During phase I of the interdigestive migrating motor complex (MMC), a total of 34 boluses of saline (0.9%) were given to each subject *via* the feeding tube. Five boluses for each volume of 1, 2 and 3 mL

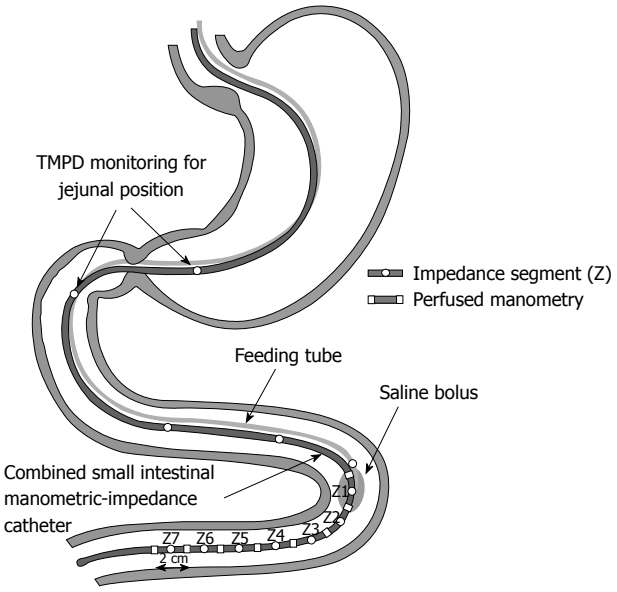


Figure 1 Outline of recording assembly, consisting of combined manometry and impedance catheters and feeding tube, positioned within the proximal small intestine.

were given at 2-min intervals. Five boluses for each volume of 5, 10 and 15 mL were given at 5-min intervals. Two boluses for each volume of 20 and 30 mL were given at 10-min interval. Each bolus was infused at the maximal rate allowed by the assembly lumen.

Data analysis

Manometry: Phase I of MMC was defined as a quiescent period without any intestinal motor activity, and phase II as a period of irregular intestinal motor activity[5,19]. A SI pressure wave was defined as a pressure rise ≥ 6 mmHg from baseline and lasting between 0.8 and 7 s[19]. Pressure waves in adjacent channels were regarded as temporally related if their onsets were within 3 s, and "propagated" if there were ≥ 3 temporally-related sequential pressure waves[19].

Impedance: The recordings were analysed manually using the impedance analysis software (Bioview Analysis, Sandhill Scientific). An impedance event judged to be associated with flow was defined as having an impedance drop of > 12% below the baseline and propagated over 3 or more impedance segments[17]. The impedance baseline for which the percentage drop was based on was the average impedance value over the 5 s immediately before the impedance drop. For each impedance event, the following variables were characterized: (1) the baseline impedance value (5 s) before injection of the bolus; (2) the magnitude of impedance drop, including minimum, average and maximum values; (3) the number of propagated channels; and (4) total bolus clearance time (TBCT), which was defined as the time taken (in seconds) for the bolus to traverse the whole recording segment in the intestine. TBCT was measured from the time the bolus entered the most proximal intestinal recording segment (Z1) until it cleared the most distal recording segment (Z7). Impedance events with TBCT greater than 60 s were classified as 'major' events (Figure 2). For each tested bolus, an impedance event was deemed to be associated with the tested bolus if the event occurred within 10 s from the administration of the bolus and an absence of corresponding motility. Boluses that had the associated impedance signal interfered by movement artefacts, or that coincided with intestinal contractions at the time of bolus administration were excluded from the final analysis as it was not possible to confidently determine whether the impedance changes in these events were related to the bolus administration, the movement artefact or coincident intestinal contractions.

Statistical analysis

Data are presented as mean ± SE. Categorical data were compared by chi-square test with Yates' correction and continuous data by Student's *t*-test, using GraphPad Prism 4 (v 4.02, San Diego, CA, USA) statistical software. The relationships between bolus volume and impedance flow events, magnitude of impedance drop, number of propagated channels and TBCT were determined by repeated measures ANOVA and Pearson's linear regression analysis. Significance was accepted at a P value < 0.05.

RESULTS

Relationship between bolus volume and impedance events

Injection of a saline bolus at the proximal end of the recording segment induced a prompt impedance drop that propagated distally. A minimum volume of 2 mL was required to induce a recognizable intestinal impedance flow event (Figure 3). As the bolus volume increased from 2 to 10 mL, the proportion of detectable intestinal impedance events increased in a linear relationship. A bolus volume of ≥ 10 mL always produced an impedance event.

Relationship between bolus volume and magnitude of impedance drop

The mean baseline impedance value was 389 ± 58 ohms. There were no significant differences in the baseline impedance values among the different bolus volumes. The mean impedance drop for a detectable flow event was 151 ± 4 ohms. The median impedance drop from baseline associated with a detectable flow event was 27% (IQR: 12%-53%). There was a weak relationship between the bolus volumes and the magnitude of impedance drop (mean: $r = 0.33$, $P < 0.0001$; minimum: $r = 0.28$, $P < 0.000$; maximum: $r = 0.30$, $P < 0.01$) (Figure 4A).

Relationship between bolus volume and number of propagated channels

Boluses with a volume of ≥ 5 mL propagated over a longer distance than those with a volume < 5 mL. Once the bolus volume was ≥ 10 mL, an impedance drop always propagated over at least 7 channels (i.e. 14 cm) (Figure 3). Up to a bolus volume of 10 mL, there was

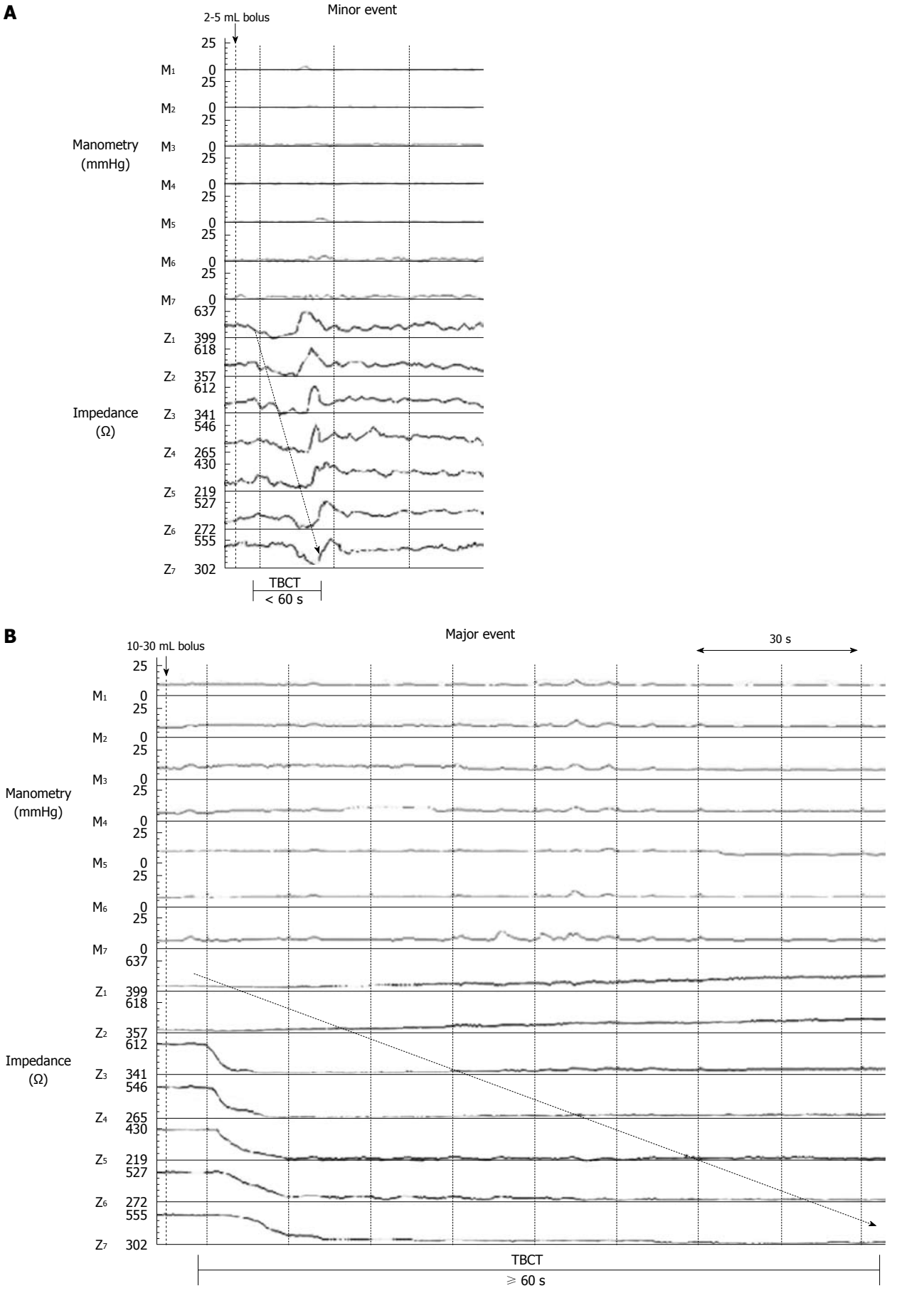


Figure 2 An example of combined manometry-impedance recording, demonstrating the patterns and the definition of total bolus clearance time (TBCT) for minor and major flow events.

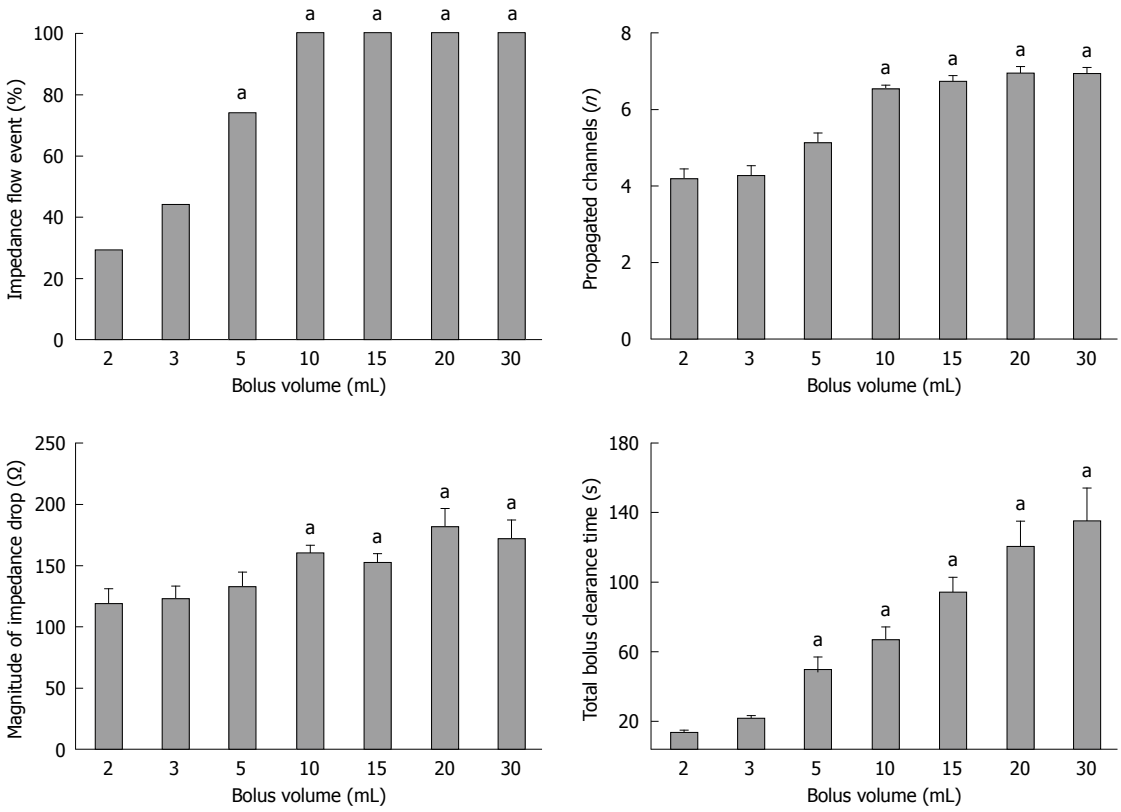


Figure 3 Relationship between bolus volume and characteristics of the associated impedance changes. [a]*P* < 0.05 *vs* 2 mL boluses.

a strong correlation between the bolus volume and the number of channels in which an impedance drop occurred ($r = 0.71$, $P < 0.00001$).

Of the administered test boluses, 48% ± 5% were followed by an intestinal pressure wave that propagated over 6.0 ± 0.1 channels. The duration between the bolus administration and the initiation of the propagated pressure wave was 20 ± 2 s. There was a positive correlation between the bolus volume and proportion of boluses followed by a propagated pressure wave ($r = 0.54$, $P < 0.0001$), which was significantly greater for bolus volumes ⩾ 15 mL (66% ± 12% *vs* 30% ± 11%, $P = 0.04$; *vs* 2 mL, respectively, Figure 4B). The bolus volume was also positively correlated with the duration between the bolus administration and the initiation of the propagated pressure wave ($r = 0.53$, $P < 0.001$).

Relationship between bolus volume and TBCT

The baseline impedance level was recovered after all bolus-induced drops, indicating bolus clearance from the impedance segment. For the majority of boluses ⩽ 5 mL, the impedance drop recovered spontaneously without an intestinal clearance pressure wave, with a TBCT of < 60 s (Figure 3). In contrast, for the majority of boluses ⩾ 10 mL, the recovery of impedance signal was more likely to require the assistance of an intestinal clearance pressure wave. Overall, there was a direct relationship between bolus volume and TBCT ($r = 0.63$, $P < 0.0001$). With increasing bolus volume, the proportion of boluses with a TBCT > 60 s also significantly increased ($P < 0.01$, Figure 4C).

DISCUSSION

This is the first methodological study to examine the relationship between bolus volume and impedance changes in the proximal small intestine. In particular, the study examined the limit of detection, and thus sensitivity, of impedance changes for identifying bolus volume. These data will enable the application of impedance as a technique to assess intestinal flow, without radiology. The main findings were (1) a bolus volume of at least 2 mL is required to generate an impedance event; (2) there is a linear relationship between bolus volume (between 2 and 10 mL) and impedance signal; (3) volumes of 10 mL or greater always generate an impedance event; and (4) bolus volume positively correlates with the magnitude of impedance drop, distance of propagation and clearance time. This modest relationship between bolus volume and intestinal impedance signals may explain, at least in part, the various patterns of flow events associated with chyme transport in the small intestine.

The threshold volume for bolus detection by impedance appears to be higher in the small intestine compared with that previously reported in the oesophagus[18]. However, the relationship between volume of liquid boluses and impedance signal observed in the proximal intestine does not exist in the oesophagus[18]. These differences

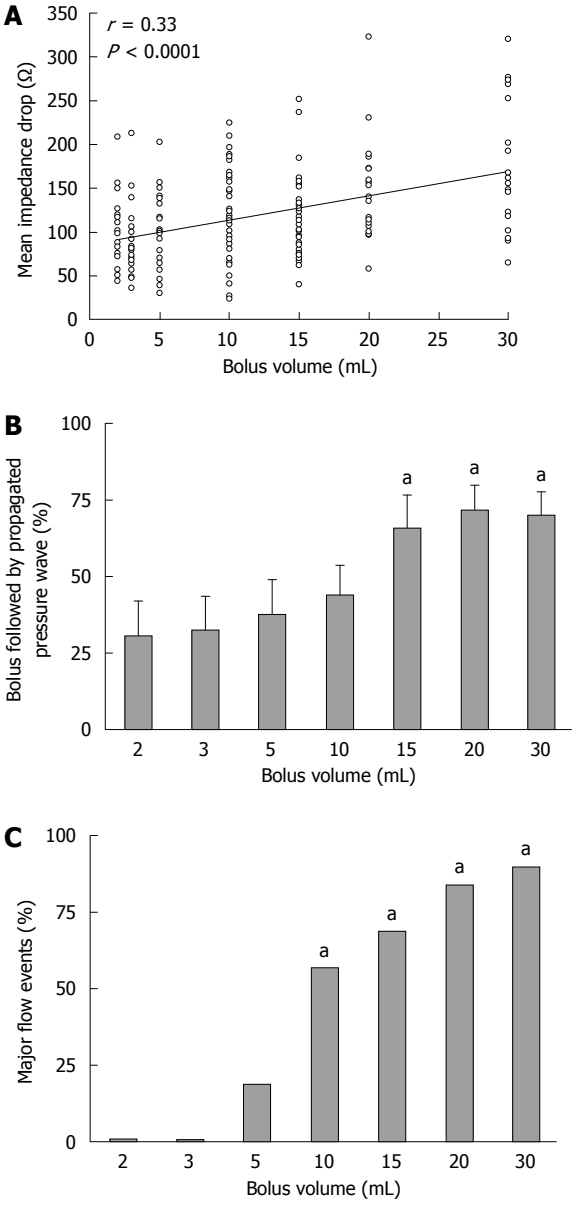


Figure 4 Relationship between bolus volume and the magnitude of impedance drop (A), the proportion of boluses followed by a propagated pressure wave (B) and the occurrence of major flow events (C). $^{a}P < 0.05$ *vs* 2 mL boluses.

may reflect structural and functional differences between the two organs[1-4,17]. The absence of mucosal secretion and possibly a more complete clearance from stronger contraction amplitudes of the oesophagus are likely to explain the higher baseline level of intra-luminal impedance in the oesophagus (approximately 1000 Ω), compared to the intestine (approximately 400 Ω)[16-18]. With a higher baseline signal, liquid boluses of 1 mL or less can induce significant and readily identifiable impedance drops in the oesophagus[18]. In contrast, with a lower baseline impedance signal, possibly caused by a relatively 'wet' lumen from constant intestinal secretions, impedance drops detected in the small intestine are proportionately smaller, and larger volumes are required to induce an identifiable impedance signal. This is supported by the current study, in which a volume threshold of 2 mL was required to induce an intestinal impedance drop. Even with this volume, impedance flow events were only observed in approximately one third of boluses, and were not observed consistently until the volume was at least 10 mL.

The characteristics of impedance changes associated with liquid transit in the small intestine were also influenced by the bolus volume. In the current study, transit of liquid boluses with a volume between 2 and 5 mL was typically associated with a shorter clearance time (less than 60 s) and propagation distance (less than 10 cm). This pattern of flow has been reported to occur during the postprandial period and possibly phase 2 of the MMC[16,17]. In contrast, transit of larger volumes (> 20 mL) were predominantly associated with clearance times greater than 60 s and propagating over 14 cm. The signals of these 'major' impedance events did not spontaneously recover to baseline level until the bolus was cleared by intestinal contraction(s). This pattern of luminal flow is typically seen at the end of phase 3 activity, which may be consistent with the clearance function of the MMC[16,17]. Characteristics of impedance events induced by liquid boluses between 10 and 20 mL varied considerably, particularly in clearance duration. This appears to depend on the timing of intestinal contraction(s) which occur after the bolus is delivered. However, there was also a direct linear relationship between the clearance time and bolus volume. The understanding of the relationship and characteristics between bolus volume and impedance changes will aid the interpretation of various patterns of luminal flow events in the small intestine.

In the current study, impedance changes associated with bolus transit were assessed in the quiescent phase of the MMC cycle, in order to avoid interference from intestinal contraction(s)[17]. Consequently, this relationship is applicable only to bolus transit within a relatively inactive small intestine. However, under normal physiological conditions of digestion, frequent intestinal contractions would be expected and may shorten the clearance time, but not propagation distance.

Furthermore, the current study used saline as a test medium, rather than chyme, which may not reflect the true transit of intestinal contents during normal digestion in humans. Nevertheless, although there are no human data that have evaluated spontaneous flow events in the small intestine, the bolus volumes used in the present study are similar to those reported during the pulsatile flow of gastric emptying in pigs[20]. However, chyme or digested food in the small intestine is typically more viscous than saline, and it is well known that viscosity significantly influences the characteristics of impedance flow events in the oesophagus[21]. Further investigation is required to assess the flow volume of chyme and its relationship with impedance signals. Due to the small diameter of the feeding tube, evaluation of a viscous medium was not possible in the current study.

A potential limitation of this study is the lack of fluoroscopic confirmation of bolus flow. However, fluoroscopy would have exposed the volunteers to excessive

radiation exposure due to the prolonged recording period. More importantly, the criteria used in this study to define an impedance "flow event" in the small intestine has already been established and validated against fluoroscopy[17]. Flow could thus be reasonably inferred from the pattern of impedance changes with evidence of propagated clearance from successive impedance segments.

In conclusion, there is a strong relationship between bolus volume and changes in impedance signals within the small intestine. Bolus volume has an impact on both the type and length of propagation of flow events and a threshold volume of 2 mL is required to produce a flow event.

COMMENTS

Background

Fluoroscopy and scintigraphy are the currently available techniques to assess intestinal motility and bolus transit, but their utilities are greatly limited by radiation exposure. Although combined intraluminal manometry and impedance has been recently used to assess flow in the small intestine, the relationship between bolus volume and impedance changes has not been assessed.

Research frontiers

The ability to use a combined intraluminal manometry-impedance technique to assess bolus transit in the small intestine will not only improve the safety of the procedure, but also its utilization in clinical practice.

Innovations and breakthroughs

This is the first methodological study to demonstrate a linear relationship between bolus volume and impedance changes in the proximal small intestine. Bolus volumes of at least 2 mL are required to generate an impedance signal that can be recognised as a flow event. Volumes of 10 mL or greater will always generate an impedance drop. These findings may explain the various patterns of flow that are associated with chyme transport in the small intestine.

Applications

These findings assist the interpretation of various patterns of bolus transit or flow of chyme in the small intestine assessed by a manometry-impedance technique, without the need for fluoroscopy or scintigraphy.

Terminology

Intra-luminal impedance is a new technique designed to detect intraluminal flow without the use of radiation. The impedance technique measures changes in resistance (in Ohms) to alternating electrical current when a bolus passes a pair of metallic rings mounted on a catheter. In an empty tubular organ (i.e. small intestine), the electrical current is conducted by the few ions present in and on the intestinal mucosa. Liquid boluses with an increased number of ions have a higher conductivity and when entering the measurement segment will lower the impedance to a nadir value. Measurement of flow at multiple sites allows for determination of bolus direction based upon temporal differences in bolus entry and exit.

Peer review

This is a high quality manuscript, presenting a series of experimental results which demonstrates, for the first time, the relationship between bolus volume and impedance changes in the proximal small intestine.

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BRIEF ARTICLE

Treatment outcome of localized *Helicobacter pylori*-negative low-grade gastric MALT lymphoma

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Abstract

AIM: To investigate treatment outcome of *Helicobacter pylori* (*H. pylori*)-negative low-grade gastric mucosa-associated lymphoid tissue (MALT) lymphoma.

METHODS: In this study, we retrospectively reviewed the clinical outcome and clinicopathologic factors of stage I E *H. pylori*-negative low-grade gastric MALT lymphoma cases from August 1998 to June 2009.

RESULTS: A total of eleven patients with *H. pylori*-negative low-grade gastric MALT lymphoma were enrolled in the study and received anti-*H. pylori* eradication treatment and/or radiotherapy or excisional therapy. Complete remission (CR) of gastric MALT lymphoma was achieved in all patients. The time to CR was 1-66 mo (median, 1 mo).

CONCLUSION: Eradication therapy may be offered as an initial treatment option even in cases of localized *H. pylori*-negative gastric MALT lymphoma.

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Key words: Anti-bacterial agents; *Helicobacter pylori*; Mucosa-associated lymphoid tissue lymphoma; Radiotherapy; Stomach

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Park HS, Kim YJ, Yang WI, Suh CO, Lee YC. Treatment outcome of localized *Helicobacter pylori*-negative low-grade gastric MALT lymphoma. *World J Gastroenterol* 2010; 16(17): 2158-2162 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i17/2158.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i17.2158

INTRODUCTION

Mucosa-associated lymphoid tissue (MALT) lymphoma, first described in 1983 by Isaacson and Wright[1], has been recently re-classified as extranodal marginal zone lymphoma of MALT-type, in the Revised European-American Classification of Lymphoid Neoplasms (REAL)/World Health Organization (WHO) Classification of Lymphoid Neoplasms. An important feature of MALT lymphoma is the presence of lymphoepithelial lesions formed by the invasion of individual glands by aggregates of lymphoma cells[2].

In previous studies, *Helicobacter pylori* (*H. pylori*) infection was suggested to be causally associated with primary gastric MALT lymphoma[3,4]. Indeed, *H. pylori* provides the antigenic stimulus, which is mediated by mucosal T cells, for sustaining the growth of gastric MALT lymphoma[5]. The regression of gastric MALT lymphoma after the eradication of *H. pylori* was first reported in 1993 by Wotherspoon *et al*[3]. *H. pylori* eradication led to complete remission in 80% of stage I E, low-grade gastric MALT lymphoma patients, with a yearly recurrence rate of approximately 5%[6]. Curr-

ently, eradication of *H. pylori* is considered the accepted initial therapy in cases of localized, stage I E low-grade gastric MALT lymphoma[7].

However, there are no treatment guidelines for the management of patients who are unresponsive to antibiotic treatment or for the subset of patients who present with stage I E low-grade gastric MALT lymphoma but are *H. pylori*-negative and understandably do not respond to antibiotic treatment[8]. In these cases, treatment choices include the known, conventional therapeutic approaches such as surgery, chemotherapy or radiotherapy[9]. Because of the ambiguity of treatment in *H. pylori*-negative patients, further studies are required to clarify a treatment strategy for *H. pylori*-negative MALT lymphoma. In this study, we evaluated the treatment outcome of eleven *H. pylori*-negative low-grade gastric MALT lymphoma patients to offer therapeutic guidelines for treating *H. pylori*-negative low-grade gastric MALT lymphoma.

MATERIALS AND METHODS

Patients

We retrospectively studied eleven patients with *H. pylori*-negative low-grade gastric MALT lymphoma diagnosed at the Severance Hospital from August 1998 to June 2009. All patients were confirmed by pathology to have low-grade gastric MALT lymphoma without a diffuse large B-cell lymphoma (DLBCL) component. Histological diagnosis of MALT lymphoma was performed according to the criteria outlined in the WHO classification, and histological assessment was performed by a reference pathologist (Yang WI). Immunological phenotyping of paraffin sections was performed to demonstrate light chain restriction and the $CD20^{+}CD5^{-}CD10^{-}cyclinD1^{-}$ phenotype which was microscopically consistent with the presence of low-grade MALT lymphoma. The study was approved by our institutional review board, and informed consent was not required.

Examination of H. pylori infection and endoscopic findings

None of the patients had evidence of *H. pylori* infection as judged by histology, a urea breath test, a rapid urease test (CLO™, Delta West, Bentley, Western Austria) and serological testing. The gross phenotype for each patient was classified into five types according to endoscopic features: (1) ulcerative: one or more ulcerations; (2) protruding: elevated or polypoid; (3) granular: small nodules on the lesion; (4) infiltrative: mucosal infiltration; and (5) mixed.

Staging work-up

Determination of the stage of disease included a detailed physical examination, chest X-ray, abdominal computed tomography (CT), endoscopic ultrasonography (EUS), bilateral bone marrow examination, and ^{18}F-FDG PET scan.

Therapeutic approach

Treatment modalities included anti-*H. pylori* eradication therapy, radiotherapy, and excisional therapy (one endoscopic mucosal resection and one subtotal gastrectomy). The most common treatment was radiotherapy (six patients), followed by anti-*H. pylori* eradication only (three patients). Prior to radiotherapy, three of the six radiotherapy patients received anti-*H. pylori* eradication therapy which consisted of amoxicillin (2 × 1000 mg/d), clarithromycin (2 × 500 mg/d), and esomeprazole (2 × 40 mg/d) or pantoprazole (2 × 20 mg/d) for 7 or 14 d. Radiotherapy was performed at a total dose ranging from 30 Gy to 36 Gy on an outpatient basis. Two patients underwent local excision therapy, including one subtotal gastrectomy and one endoscopic mucosal resection (EMR).

Response assessment

The median time for follow-up after remission was 25 mo (range: 5-76 mo). Complete remission (CR) was defined as the total disappearance of clinical evidence for lymphoma and an absence of histologic evidence for lymphoma on biopsy specimens. Partial remission (PR) was defined as a tumor reduction of at least 50%, and stable disease (SD) was defined as variation within either a 50% decrease or 25% increase in tumor size. In cases with complete remission, endoscopic examinations and biopsies were performed at regular intervals.

RESULTS

Baseline characteristics

The male to female patient ratio in this study was 1:1.2. The mean age of the patients was 55.7 (36-73) years. Four patients were symptomatic at presentation indicating abdominal pain (two cases), abdominal discomfort (one case), and GI bleeding (one case). Lymphoma was most often localized in the body and the antrum in 36% of the patients. Endoscopic lesions were characterized as ulcerative (five cases), infiltrative (one case), protruding (two cases), granular (one case) and mixed (two cases). Initial endoscopic findings are summarized in Table 1. Initial clinical staging with EUS and/or CT scans and BM examinations revealed all the cases to be stage I E.

Treatment outcome

Complete remission (CR) of gastric MALT lymphoma was achieved in all patients (Table 2). The time to CR ranged from one to 66 mo (median, 1 mo). Anti-*H. pylori* eradication therapy was performed in six of the eleven patients. Three of six patients, who completed the follow-up endoscopic examination 1 or 2 mo later, had complete remission of gastric MALT lymphoma. However, two patients refused to wait for the treatment response evaluation and subsequently underwent radiotherapy for definitive treatment. The remaining patient had stable disease for 2 mo before being referred for radiotherapy. All three of these patients showed complete remission of gastric MALT lymphoma 1 mo after the cessation of radiotherapy based on histological evidence. None of the 11 patients showed

Table 1 Baseline characteristics of *H. pylori*-negative low-grade gastric MALT lymphoma *n* (%)

	H. pylori negative MALT lymphoma (*n* = 11)
Age (yr, mean)	55.7 (36-73)
Sex	
Men	5 (45)
Women	6 (55)
Site	
Cardia	1 (9)
Fundus	1 (9)
Body only	3 (27)
Body & antrum	4 (36)
Diffuse	2 (18)
Endoscopy	
Ulcerative	5 (45)
Infiltrative	1 (9)
Protruding	2 (18)
Granular	1 (9)
Mixed	2 (18)

H. pylori: *Helicobacter pylori*; MALT: Mucosa-associated lymphoid tissue.

Table 2 Individual characteristics of 11 cases of *H. pylori*-negative low-grade gastric MALT lymphoma

	Age (yr)/ sex	Stage	Treatment	Response	Time to CR (mo)	Follow up time after remission (mo)
Case 1	54/F	I E	Eradication	CR	1	6
Case 2	60/F	I E	Eradication	CR	1	27
Case 3	52/F	I E	Eradication	CR	2	25
Case 4	36/M	I E	RTx	CR	1	76
Case 5	58/F	I E	RTx	CR	2	17
Case 6	69/M	I E	RTx	CR	1	47
Case 7	41/F	I E	Eradication + RTx	CR	1	7
Case 8	62/M	I E	Eradication + RTx	CR	1	8
Case 9	55/M	I E	Eradication -> RTx	CR	1	5
Case 10	73/F	I E	STG	CR	66	54
Case 11	53/M	I E	EMR	CR	2	69

CR: Complete remission; RTx: Radiotherapy; STG: Subtotal gastrectomy; EMR: Endoscopic mucosal resection.

local or distant recurrence after a median follow-up time of 25 mo (range: 5-76 mo).

DISCUSSION

The prevalence of *H. pylori* in patients with low-grade gastric MALT lymphoma is variable[10]. It is possible that a reduced number of *H. pylori* organisms present in the infection may account for the negative *H. pylori* diagnostic test result in some cases, and false-negative results may also be obtained when only one diagnostic method is employed[11]. The European Guidelines generally take the gold standard to be represented by at least two tests[12]. When appropriate diagnostic methods are used, the prevalence of *H. pylori* infection in low-grade MALT lymphoma is high, at nearly 90%[10]. In our study, four diagnostic tests were performed and showed that true *H. pylori*-negative patients were included. However, additional tests, such as culture with polymerase chain reaction, would allow the detection and identification of other infective organism[13].

Due to the excellent clinical outcome of *H. pylori* eradication treatment, the eradication of *H. pylori* with antibiotics should be employed as the sole initial treatment in the localized form (confined to the stomach) of gastric MALT lymphoma[14]. However, in advanced stages with *H. pylori*-positive gastric MALT lymphoma and in *H. pylori*-negative lymphoma, definitive treatment guidelines are not yet available despite the numerous published clinical research papers[8,14,15].

Antibiotic treatment for *H. pylori*-negative gastric MALT lymphoma has been described in a limited number of patients. We collectively reviewed a series of four published studies including patients treated solely with the anti-*H. pylori* antibiotic regimen as the initial treatment for *H. pylori*-negative stage I E gastric MALT lymphoma (Table 3). A total of 31 patients were included in these studies, and ten patients (32%) were shown to respond to this treatment alone. Raderer *et al*[16] reported that five of six patients with *H. pylori*-negative gastric MALT lymphoma responded to antibiotic treatment (one partial remission and four complete remissions). However, Steinbach *et al*[17] and Ye *et al*[18] experienced a 0% response rate. In other words, marked variation in treatment response rate was noted. Nevertheless, instituting anti-*H. pylori* treatment showed satisfactory clinical outcomes and allowed for gastric preservation in a significant number of cases with *H. pylori*-negative gastric MALT lymphoma. Although seemingly contradictory, anti-*H. pylori* treatment may be advised as the first-line therapy in *H. pylori*-negative localized gastric MALT lymphoma. Unfortunately, the effectiveness of anti-*H. pylori* treatment could not be confirmed in this study, as only four out of the six patients who received the anti-*H. pylori* eradication regimen waited long enough to receive the follow-up endoscopic examination which was used to confirm the complete remission status of MALT lymphoma. The other two patients proceeded to radiation therapy due to various clinical and psychosocial issues. In addition, previous studies also included patients with advanced stages of disease, including ⅡE$_1$ (Table 3). However, it was not possible from the available data to categorize lymphoma regression according to tumor stage (I E *vs* ⅡE$_1$). Therefore, additional studies are needed to define the role of anti-*H. pylori* eradication therapy in advanced stages of gastric MALT lymphoma such as stages ⅡE, ⅢE or Ⅳ.

There is still no explanation for the effectiveness of anti-*H. pylori* treatment in *H. pylori*-negative gastric MALT lymphoma[16]. However, it has been speculated that another infective organism, *Helicobacter heilmannii*[19], might be involved in the development of gastric MALT lymphoma. Also, one cannot rule out the possibility that unknown bacterial agents capable of surviving in the stomach might play a role in the development of rare *H. pylori*-negative

Table 3 Efficacy of eradication treatment in *H. pylori*-negative gastric MALT lymphoma reported in the literature *n* (%)

Author	Yr	No. of patients	Stage	Follow-up after treatment (mo)	Response rate
Steinbach *et al*[17]	1999	6	I E	5 or more	0 (0)
Ye *et al*[18]	2003	5	I E	4-12	0 (0)
Raderer *et al*[16]	2006	6	I E	12-19	5 (83)
Stathis *et al*[23]	2009	14	I E	Not described	5[1] (35)
Ruskone-Fourmestraux *et al*[24]	2001	10	I E, II E$_1$	2-21	0 (0)
Nakamura *et al*[25]	2006	7	I E, II E$_1$	1-15	2 (29)
Akamatsu *et al*[21]	2006	9	I E, II E$_1$	6 or more	1 (11)
Terai *et al*[26]	2008	4	I E, II E$_1$	Not described	1 (25)

[1]3 presented a local relapse.

Table 4 Efficacy of radiotherapy for *H. pylori*-negative gastric MALT lymphoma reported in the literature

Author	Yr	No. of patients	Stage	Follow-up after treatment, median (mo)	Response rate (%)
Schechter *et al*[27]	1998	12	I E, II E	Not described	100
Ye *et al*[18]	2003	1[1]	I E	12	100
Akamatsu *et al*[21]	2006	12 (5[1])	I E, II E$_1$	Not described	100
Chung *et al*[22]	2009	4	I E, II E	12-39	100

[1]Failure of anti-*H. pylori* treatment as the initial therapy.

gastric MALT lymphomas[16]. Another possibility is that low bacterial counts[20] and urease-negative *H. pylori* mutant strains may escape detection by the diagnostic tests that are currently available. If this assumption is correct, one might expect the noted *H. pylori*-negative gastric MALT lymphoma patient response to broad-spectrum antibiotic therapy. Also, MALT lymphoma is to some extent an immunologically driven disease, therefore an additional hypothesis may involve the potential immunomodulatory effects of the antibiotic agents used[16].

Disease control using radiotherapy alone has been previously reported in the literature, which supports the use of a modest dose of involved-field radiotherapy for patients with stages I E-II E MALT lymphoma of the stomach without evidence of *H. pylori* infection[14]. Table 4 summarizes these previously reported cases. Including our cases, 35 of 35 cases responded to radiotherapy. One of the 35 patients reported by Akamatsu *et al*[21] had a partial remission, while the others all had a complete remission. Based on these results, it is prudent to say that radiotherapy is suitable in early stage (I E or II E) *H. pylori*-negative gastric MALT lymphoma. In addition, excisional treatments such as endoscopic mucosal resection or laparoscopic gastric resection may represent another therapeutic option in the case of localized forms of gastric MALT lymphoma in individuals who are not suitable for, or who refuse, standard therapeutic approaches[15,20,22]. In the past, surgical treatment has often been advocated to establish accurate diagnosis, staging, and management of early-stage gastric lymphoma, which has about a 90% 5-year survival rate, but leads to significant morbidity[22]. In our study, two patients received endoscopic mucosal resection and subtotal gastrectomy, respectively. There has been no local or distant recurrence during the follow-up period of 54 to 69 mo.

In our series, therapeutic measures such as anti-*H. pylori* eradication, radiotherapy and excisional therapy achieved complete remission in all cases. Nevertheless, antibiotic treatment is simple, inexpensive, less harmful and *H. pylori* negative low grade MALT lymphoma shows a favorable long-term outcome[22]. Therefore we suggest that *H. pylori* eradication therapy may be an initial treatment option for localized *H. pylori*-negative gastric MALT lymphoma. In addition, further studies on eradication therapy are required to help in the establishment of strategies for patients with localized *H. pylori*-negative gastric MALT lymphoma.

COMMENTS

Background

Eradication of *Helicobacter pylori* (*H. pylori*) is a well-accepted initial therapy in cases of localized (stage I E) low-grade gastric mucosa-associated lymphoid tissue (MALT) lymphoma associated with *H. pylori* infection. However, there are no treatment guidelines for the management of *H. pylori*-negative low-grade gastric MALT lymphoma.

Research frontiers

Previous studies revealed the effectiveness of radiotherapy for localized *H. pylori*-negative low grade gastric MALT lymphoma. However, *H. pylori* eradication therapy is still a controversial treatment modality, although this treatment is simple and less harmful than other treatments.

Innovations and breakthroughs

With the exception of this study, only 31 *H. pylori*-negative patients have received eradication therapy (response rate 32%). However, treatment response was variable. The authors' study enrolled a small number of patients, but they all had complete remission.

Applications

The findings from this study suggest that *H. pylori* eradication therapy may be an initial treatment option for localized *H. pylori*-negative gastric MALT lymphoma. Their study adds information which helps in the establishment of strategies for patients with localized *H. pylori*-negative gastric MALT lymphoma.

Peer review

This is an interesting paper that may be beneficial for patients with *H.pylori*-negative gastric MALT lymphoma.

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BRIEF ARTICLE

Serum VEGFR-3 and survival of advanced gastric cancer patients treated with FOLFOX

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Abstract

AIM: To explore if vascular endothelial growth factor receptor-3 (VEGFR-3) and carcinoembryonic antigen (CEA) can predict overall survival in advanced gastric cancer.

METHODS: VEGFR-3 level was assessed by enzyme-linked immunosorbent assay, and CEA was assessed by chemiluminescence immunoassay in the sera of 81 advanced gastric cancer patients before treatment with oxaliplatin plus 5-fluorouracil and folinic acid.

RESULTS: Median survival time in patients with a low serum VEGFR-3 level was significantly longer than in those with a higher VEGFR-3 level (15.4 mo *vs* 7.7 mo, $P < 0.001$). Patients with a low CEA level had a longer survival than those with a higher CEA level (15.8 mo *vs* 8.6 mo, $P < 0.001$). Thirty-nine patients with low VEGFR-3 and low CEA levels had a median survival of 19.7 mo ($P = 0.0006$). The hazard ratio for patients with a high VEGFR-3 level was 2.443 ($P = 0.002$).

CONCLUSION: High serum VEGFR-3 level is correlated significantly with poor survival. In patients with a high serum level of VEGFR-3, alternative chemotherapy regimens should be considered.

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Key words: Stomach neoplasms; Vascular endothelial growth factor receptor-3; Carcinoembryonic antigen; Oxaliplatin plus 5-fluorouracil and folinic acid protocol; Survival

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INTRODUCTION

According to global estimates, gastric cancer is the second most frequent cancer-related cause of death. The incidence of gastric cancer is estimated to be 934 000 cases, with 56% of the new cases occurring in East Asia, 41% in China and 11% in Japan[1]. In 2005, there were approximately 400 000 new cases and 300 000 deaths from gastric cancer in China[2].

Great efforts are being made to develop serological markers that are noninvasive and can easily reflect the dynamic status of the tumor. Among these, carcinoembryonic antigen (CEA) has been used widely as a serological marker in patients with gastrointestinal malignancies[3-5]. CEA was first described by Gold and Freedman in 1965 as an antigen that is expressed by gastrointestinal carcinoma cells, which is secreted in blood or body fluids. Several studies have focused on the utility of CEA and carbohydrate antigen 19-9 (CA19-9) measurement in cancer progression, recurrence, and prognosis in patients with gastric carcinoma[4,6,7].

There has been a dramatic increase in the number of studies of the mechanisms of associated lymphangio-

genesis and lymphatic metastasis. It has been recognized that lymphangiogenic growth factors promote the spread of cancer cells to regional lymph nodes[8-10], and one of the most important ones is vascular endothelial growth factor receptors (VEGFR).

Regional lymph node metastasis is an important indicator of tumor aggressiveness, as well as a known prognostic factor[11]. Therefore, it is important to estimate the degree of lymphatic system invasion and lymphangiogenesis in the evaluation of biological tumor aggressiveness and patient outcome. VEGFRs1, 2 and 3 are endothelial-specific receptor tyrosine kinases that are regulated by members of the VEGF family. VEGFR-3 expression has been demonstrated in a variety of human malignancies[12]. The role of the VEGF-C, D and/or VEGFR-3 axis in various types of cancer has been investigated by many research groups[12]. In clinical studies, a negative correlation between VEGF-C, D and/or VEGFR-3 and patient survival time has been reported in non-small cell lung cancer, colorectal carcinoma, endometrial carcinoma, epithelial ovarian carcinoma and primary breast cancer[13-17].

Advanced gastric cancer patients need chemotherapy, but there is currently no established standard regimen; oxaliplatin plus 5-fluorouracil and folinic acid (FOLFOX) is well tolerated. Recently, several phase Ⅱ studies have yielded a median time to progression (TTP) of 5.4-6.5 mo and a median overall survival (OS) of 9.8-12.6 mo[18-21].

A reliable factor is necessary to predict objectively the effectiveness of some special chemotherapy protocols. Recently, two studies have investigated if gene polymorphisms and mRNA can predict the TTP and OS in advanced gastric cancer treated with FOLFOX[22,23]. For oxaliplatin-associated chemotherapy of advanced gastric cancer, seeking more reliable predictive values for chemotherapy is of great importance in research and clinical settings.

We hypothesized that secreted CEA reflects the tumor burden, and VEGFR-3 is associated with poor survival. The purpose of this study is to find out the prognostic value of serum levels of VEGFR-3 and CEA in gastric cancer patients receiving FOLFOX chemotherapy.

MATERIALS AND METHODS

Patients

Patients with histologically proven locally advanced or metastatic gastric cancer and Eastern Cooperative Oncology Group (ECOG) performance status (PS) $\leqslant 2$ were included in the study. Clinical stage and histological type of gastric cancer were evaluated according to AJCC criteria (the sixth edition). All patients received FOLFOX chemotherapy after resection of primary tumors as follows: oxaliplatin 130 mg/m^2 on day 1, plus folinic acid 200 mg/m^2 as a 2-h infusion, followed by a 22-h infusion of 5-fluorouracil (5-FU) 450 mg/m^2 on days 1-5, every 3 wk. Survival was calculated from the date of diagnosis to the date of last follow-up or death from any cause.

All patients gave their signed informed consent, and the study was approved by the Institutional Ethics Review Boards.

Quantitative detection of VEGFR-3 and CEA

Sera from 40 healthy volunteers (20 females and 20 males ranging in age from 25 to 60 years) and gastric cancer patients were collected using a serum separator tube and kept frozen at -80℃ until assay.

Enzyme-linked immunosorbent assay for serum VEGFR-3 and CEA

Serum was collected before chemotherapy. VEGFR-3 was analyzed with enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA). An ELISA component kit that measures the extracellular (soluble) domain of VEGFR-3 was employed. Serum VEGFR-3 assays were calibrated against recombinant proteins that consisted of the full-length extracellular domain of the respective receptors. A 96-well microplate was coated with diluted capture antibody, and incubated overnight. After washing, the plate was blocked by adding diluent reagent. Plate preparation was finished. Samples or standards were added, then the plates were washed, detection antibody was added, and washing was repeated. Streptavidin-horseradish peroxidase was added to each well. After washing, substrate solution was added to each well. Finally, stop solution was added to each well. The plate was tapped gently. The optical densities of each well were quantified within 30 min at dual wavelengths of 450 nm corrected to 540 nm using a micro-plate reader.

CEA level of all serum samples was analyzed by chemiluminescence immunoassay (CEA Regent Kit, Abbott Diagnostics). Assays were carried out according to the manufacturer's instructions using the machine of ARCHITECT i2000 SR.

Statistical analysis

Spearman's Rho method was used to correlate levels of VEGFR-3 and CEA. The maximal χ^2 method of Miller *et al*[24] and Halpern[25] was adapted to determine which cutoff value can best dichotomize the patients into low- and high-expression CEA and VEGFR-3 subgroups; the Tree method[26] was then applied to optimize these cutoff values. The final cutoff values were confirmed by recursive partitioning and amalgamation using S-Plus software, version 6.1 (Statistical Sciences, Seattle, WA, USA). Cumulative survival rates were determined using the Kaplan-Meier method, and the difference between each group was evaluated by the log-rank method. The cases lost to follow-up were treated as censored data for the analysis of survival rates. A univariate Cox model with overall survival as the dependent variable was constructed and categorized with two factors levels as independent variables, and the factors that were significant in the univariate analysis were included in a multivariate Cox proportional hazards model for survival. Differences were considered significant at $P < 0.05$. All statistical calculations were performed using the Statistical Package for the Social Sciences, version 13 (SPSS Inc., Chicago, IL, USA).

Table 1 Clinical characteristics associated with overall survival of the patients

Characteristics	*n* (%)	MST (mo) (95% CI)	*P* Log-rank test
Age (yr) (median: 59, range: 28-73)			
< 59	37 (45.7)	13.900 (8.075-19.725)	0.610
⩾ 59	44 (54.3)	12.600 (6.595-18.605)	
Gender			
Male	55 (67.9)	12.600 (7.674-17.526)	0.384
Female	26 (32.1)	15.300 (6.017-24.583)	
ECOG			
0-1	64 (79.0)	14.300 (9.194-19.406)	0.034
2	17 (21.0)	9.800 (5.307-14.293)	
Lymph node			
Negative	13 (16.0)	35.100 (5.695-64.505)	0.004
Positive	68 (84.0)	9.900 (7.480-12.320)	
Initial staging			
Ⅲ	38 (46.9)	17.800 (14.187-21.413)	0.017
Ⅳ	43 (53.1)	8.600 (6.157-11.043)	
Grading			
G2	23 (28.4)	14.500 (2.761-26.239)	0.584
G3	58 (71.6)	12.600 (7.823-17.377)	
Site of tumor			
Proximal stomach	30 (37.0)	10.300 (5.283-15.317)	0.806
Distal stomach	44 (54.3)	15.400 (9.380-21.420)	
Whole stomach	7 (8.6)	10.800 (4.439-17.161)	

MST: Median survival time; ECOG: Eastern Cooperative Oncology Group.

RESULTS

Patient characteristics

A total of 81 advanced gastric adenocarcinoma patients were included in the study. The median age was 59 years; 55 patients were male and 64 patients had ECOG PS 0-1; and 13 patients had no lymph node involvement. Thirty-eight (46.9%) patients had stage Ⅲ, and 43 (53.1%) had stage Ⅳ disease at the time of diagnosis. Patient characteristics are summarized in Table 1.

Levels of VEGFR-3 and CEA

Serum VEGFR-3 and CEA levels were detected in all the patients and healthy donors. Serum VEGFR-3 level varied in healthy donors, and the median level was 24.5 ng/mL [range: 0.3-40.3 ng/mL, 95% confidence interval (CI): 19.2-31.7]. In gastric cancer, the median VEGFR-3 level was 41.8 ng/mL (range: 17.2-385.5 ng/mL, 95% CI: 52.5-114.6). A highly significant difference was found in the median VEGFR-3 level between gastric cancer patients ($P < 0.01$) and healthy donors.

Serum CEA level was variable in healthy donors, and median level was 2.3 ng/mL (range: 0.1-6.2 ng/mL, 95% CI: 1.7-3.9). In gastric cancer, the median CEA level was 13.7 ng/mL (range: 2.2-301.7 ng/mL, 95% CI: 18.3-57.8). A highly significant difference was found in the median CEA level between gastric cancer patients ($P < 0.01$) and healthy donors.

Using a cutoff value of 70.6, 52 (64.2%) patients had low VEGFR-3 expression levels and 29 (35.8%) patients had higher VEGFR-3 levels. Using a cutoff value of 23.5, 57 (70.4%) patients had low CEA expression levels and 24 (29.6%) had higher levels. There was no significant association between VEGFR-3 and CEA levels ($P = -0.136$, $P = 0.227$). No significant association was detected between VEGFR-3 or CEA and clinical parameters.

Table 2 Serum VEGFR-3 and CEA levels and survival in advanced gastric cancer patients

Factors	*n*	MST (mo) (95% CI)	*P*[1]
VEGFR-3 (ng/mL)			
Low ⩽ 70.6	52	15.400 (11.799-19.001)	< 0.001
High > 70.6	29	7.700 (5.691-9.709)	
CEA (ng/mL)			
Low ⩽ 23.5	57	15.800 (11.223-20.377)	< 0.001
High > 23.5	24	8.600 (7.219-9.981)	

[1]Adjusted *P*-value based on log-rank statistics after 1000 bootstrap simulations. VEGFR-3: Vascular endothelial growth factor receptor-3; CEA: Carcinoembryonic antigen; CI: Confidence interval.

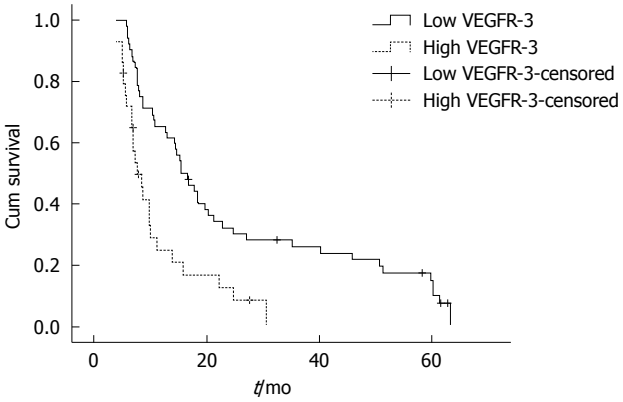


Figure 1 Kaplan-Meier estimates of overall survival by vascular endothelial growth factor receptor-3 (VEGFR-3) levels (*n* = 81, low VEGFR-3: 52; high VEGFR-3: 29).

Survival time

The median survival time for all patients was 13.0 mo (95% CI: 8.820-17.180). A significant association was observed between survival and ECOG PS ($P = 0.034$), lymph node involvement ($P = 0.004$) and initial staging ($P = 0.017$). No other association between clinical characteristics and survival was found (Table 1).

A significant association was also observed between survival and levels of VEGFR-3 ($P < 0.05$) and CEA ($P < 0.05$). Median survival for patients with low VEGFR-3 levels was 15.4 mo (95% CI: 11.799-19.001) compared with 7.7 mo (95% CI: 5.691-9.709) for those with higher VEGFR-3 levels ($P < 0.001$) (Table 2 and Figure 1). Median survival for patients with low CEA levels was 15.8 mo (95% CI: 11.223-20.377) compared with 8.6 mo (95% CI: 7.219-9.981) for those with higher CEA levels ($P < 0.001$, Table 2).

Among the 24 patients with high CEA levels, 13 patients with low VEGFR-3 levels had a median survival of 13.0 mo (95% CI: 5.954-20.046) while 11 patients with higher VEGFR-3 levels had a median survival of 7.2 mo (95% CI: 4.622-9.778) ($P = 0.003$, Figure 2A).

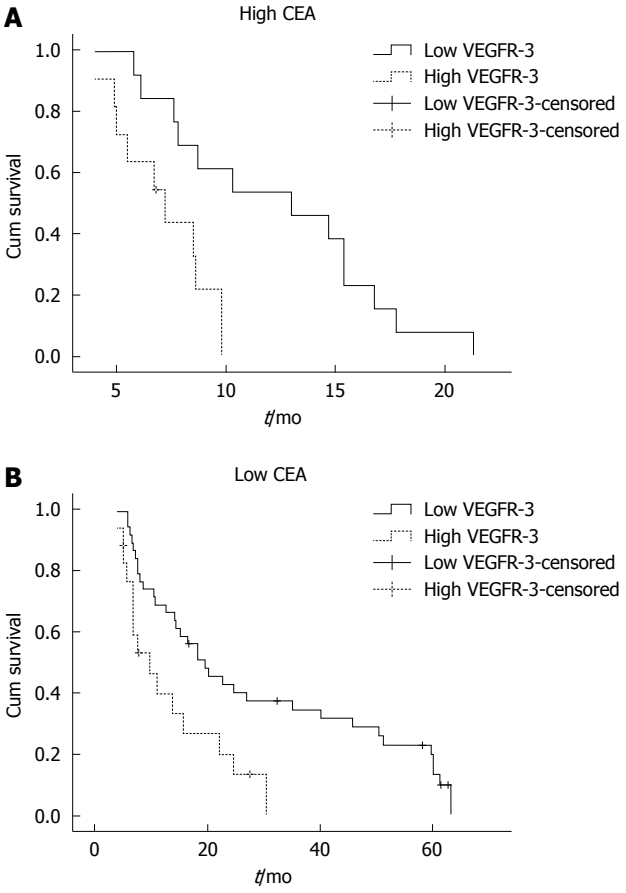


Figure 2 Kaplan-Meier estimates of overall survival according to VEGFR-3 levels in patients. A: Patients with high carcinoembryonic antigen (CEA) levels (n = 24, high CEA and low VEGFR-3: 13, high CEA and high VEGFR-3: 11); B: Patients with low CEA levels (n = 57; low CEA and low VEGFR-3: 39; low CEA and high VEGFR-3: 18).

Among the 57 patients with low CEA levels, 39 patients with low VEGFR-3 levels had a median survival of 19.7 mo (95% CI: 12.53-26.87) while 18 patients with higher VEGFR-3 levels had a median survival of 9.9 mo (95% CI: 4.651-15.149) (P = 0.006, Figure 2B).

Multivariate analysis identified VEGFR-3 levels [hazard ratio (HR) = 2.443, P = 0.002], initial staging (HR = 1.844, P = 0.018), and ECOG PS (HR = 2.396, P = 0.011) as independent markers for survival (Table 3), whereas CEA levels (HR = 1.255, 95% CI: 0.721-2.184, P = 0.121) were not an independent marker for survival.

DISCUSSION

In this study of serum levels of VEGFR-3 and CEA in advanced gastric cancer, multivariate analysis identified VEGFR-3 levels, initial staging, and ECOG PS as independent markers for the survival of the patients who received the FOLFOX regimen. CEA was not an independent marker for survival.

VEGF-C and VEGFR-3 are associated with lymphatic metastasis, mainly *via* tumor lymphangiogenesis in animal models and human tumors[9,27]. Many previous studies have investigated the association between expression of VEGFR-3 and tumor histology. VEGFR-3 is an independent prognostic factor for survival; the expression of VEGFR-3 is correlated with lymphatic invasion, lymph node metastasis, and poor prognosis for survival[28-30]. In the present study, the median survival time in patients with low serum VEGFR-3 levels was significantly longer than in those with higher VEGFR-3 levels (15.4 mo *vs* 7.7 mo, $P < 0.001$). Patients with low VEGFR-3 and CEA levels had a median survival of 19.7 mo (P = 0.0006). The HR for patients with a high VEGFR-3 level was 2.443 (P = 0.002).

Table 3 Multivariate analysis of factors associated with overall survival

Factors	*n*	Hazard ratio (95% CI)	*P*
ECOG PS			
0-1	64	1 (ref.)	
2	17	2.396 (1.219-4.708)	0.011
Lymph node			
Negative	13	1 (ref.)	
Positive	68	1.846 (0.871-3.912)	0.110
Initial staging			
Ⅲ	38	1 (ref.)	
Ⅳ	43	1.844 (1.109-3.066)	0.018
VEGFR3			
Low ≤ 70.6	52	1 (ref.)	
High > 70.6	29	2.443 (1.374-4.345)	0.002

PS: Performance status.

Although great efforts have been devoted to improve early detection of gastric cancer, the majority of patients are diagnosed at an advanced stage. The median OS has been shown to be 9.8-12.6 mo after FOLFOX chemotherapy[18-21]. Identification of patients with potentially poor prognosis after FOLFOX chemotherapy would help us to optimize another treatment protocol for patients with advanced gastric cancer.

These findings are similar to those with metastatic malignant melanoma, for which, a high pretreatment serum VEGFR-3 level is correlated significantly with poor prognosis. Patients with a low serum VEGFR-3 level had a higher median disease-free survival than those with a high serum VEGFR-3 level (16.2 mo *vs* 10.8 mo, χ^2 = 3.85, P = 0.022). Median serum VEGFR-3 levels were significantly higher in patients with a high tumor burden than those with a low tumor burden (P = 0.013)[31].

There are few reports on biomarkers with a high and reliable predictive value for chemotherapy. Recently, two studies have investigated predictive factors for FOLFOX chemotherapy in advanced gastric cancer. In one study of genetic polymorphism, the glutathione S-transferase M1 positive genotype showed a significantly longer survival time compared with negative genotype in advanced gastric cancer treated with FOLFOX[22]. In a study of mRNA, the median survival time in patients with low levels of mammalian excision repair *via* complementing protein ERCC1 was significantly longer than in those with higher levels (15.8 mo *vs* 6.2 mo, $P < 0.0001$) in advanced gastric cancer treated with FOLFOX chemotherapy[23].

However, in the present study, the difference in the survival of the patients with a low level and a high level of VEGFR-3 was much more striking (15.4 mo *vs* 7.7 mo, $P < 0.001$), with an HR of 2.443. The determination of relative serum VEGFR levels by ELISA and electrochemiluminescence is considered currently to be easier than by immunohistochemistry and quantitative PCR.

It has been shown that VEGFR-3 is expressed not only in lymphatic endothelial cells, but also in tumor cells, and it has been seen in the cytoplasm, along the nuclear and cell membranes, which underlines its potential role in tumor growth[15,32,33]. Despite vast amounts of literature on VEGFR-3 expression in tissues (quantitative PCR and immunohistology)[32,34-37], there are few data about serum levels of VEGFR-3, which is the major axis specific for lymphangiogenesis.

To address the biological and clinical significance of pre-chemotherapeutic serum VEGFR-3 levels in advanced gastric cancer, we compared serum VEGFR-3 levels with clinicopathological parameters. A significant association was observed between survival and ECOG PS, lymph node involvement and initial staging. We found a significant association between the pre-chemotherapeutic serum level of VEGFR-3 and lymph node status. In contrast, there was no correlation between survival and age, sex, tumor grading, or tumor site.

In a recent study of anti-angiogenic treatment, DePrimo *et al*[38] found that the decrease in the soluble variant of VEGFR-3 could be a marker of sunitinib activity in patients with metastatic renal cell carcinoma. Similar to our results, Rini *et al*[39] have shown that a high baseline VEGFR-3 level is related to non-response to treatment and shorter progression-free survival in renal cell cancer that is refractory to bevacizumab, when the patients are treated with sunitinib. The presence of high levels of circulating VEGFR-3 in advanced gastric cancer patients might prospectively identify high-risk patients undergoing FOLFOX chemotherapy with a worse prognosis and shorter survival, and special target medicine is needed for therapy.

It is also not known in which way VEGFR-3 contributes to tumor angiogenesis, lymphangiogenesis, tumor progression and metastasis[40,41]. However, our results were from serum, and further investigations are necessary to confirm our observations.

A high CEA level has been shown to be a negative factor for survival in breast, gastric and colorectal cancer[42-47]. In patients with metastatic colorectal cancer receiving cetuximab plus FOLFIRI or FOLFOX-4 chemotherapy, serum CEA could predict progression-free survival time. Survival time in responders assessed by changes in serum CEA was significantly longer than that in non-responders ($P = 0.0091$)[48].

In another study, a preoperative CEA level was an independent prognostic factor in patients with node-positive Dukes' C colorectal cancer treated with 5-FU-based adjuvant chemotherapy[49]. However, the role of CEA in predicting chemosensitivity remains controversial. In a prospective study, by multivariate analysis, serum CA19-9 level ($P < 0.001$) was found to be an independent prognostic factor, whereas pretreatment serum CEA level was not considered to be a significant prognostic indicator in patients with metastatic colorectal cancer treated with 5-FU-based chemotherapy[50].

In the present study, patients with lower CEA levels had a longer survival than those with higher CEA levels, especially in patients with higher VEGFR-3 levels. VEGFR-3 over-expression increases proliferation of MCF7 cells, but proliferation of BT474 cells is reduced drastically when endogenous VEGFR-3 is down-regulated[51]. Producing a plausible explanation of why a meaningful number of patients with low VEGFR-3 and CEA levels had the longest survival is one of the aims of the present study.

Irinotecan and taxane-based regimens have been used in the treatment of advanced gastric cancer patients, with a similar survival to those attained with FOLFOX[52-54]. Irinotecan or taxane-based regimens could be the better alternative for patients with high VEGFR-3 levels. A randomized customized trial is warranted in this setting.

Targeting the VEGF-C/VEGFR-3 axis may be therapeutically significant for certain types of tumors. Thus, the continued discovery and characterization of factors that regulate VEGF-C or VEGFR-3 are essential for developing new therapies that limit the spread of cancer. In a recent study of gastric tumors, the target drug Ki23057 inhibited the phosphorylation of VEGFR-3 in lymphatic endothelial cells. The degree of lymphatic invasion and lymphangiogenesis was significantly ($P < 0.05$) lower in the gastric tumors treated by Ki23057[55]. Therefore, the target medicine is required to be developed in the future. In particular, new drugs that block the VEGFC/VEGFR-3 signaling pathway may provide useful anticancer therapeutics by mechanisms other than the blockage of lymphangiogenesis.

In conclusion, the data from our study indicate that serum VEGFR-3 level is the most significant prognostic indicator of patients with advanced gastric cancer. It is recommended that stratification for further clinical trials of patients with advanced gastric cancer should be carried out according to serum VEGFR-3 levels. Combined analysis of the VEGF-C/VEGF-D/VEGFR-3 system might be useful for identifying patients with an unfavorable clinical outcome, thereby helping refine therapeutic decisions in gastric cancer.

In our study, we only detected the levels of VEGFR-3 and CEA before chemotherapy. Changes in these levels after chemotherapy, especially with regard to differences between the responding and non-responding groups, need further researches.

COMMENTS

Background

Carcinoembryonic antigen (CEA) has been used widely as a serological marker in patients with gastrointestinal malignancies. Several reports have focused on the utility of CEA measurements in cancer progression, recurrence, and prognosis in patients with gastric carcinoma. Vascular endothelial growth factor

receptor-3 (VEGFR-3) expression has been demonstrated in a variety of human malignancies. CEA reflects the tumor burden, and VEGFR-3 is associated with tumor progression. For oxaliplatin-associated chemotherapy of advanced gastric cancer, seeking a more reliable predictive marker for chemotherapy has been of great interest in research and clinical settings.

Research frontiers

In this study, serum VEGFR-3 and CEA levels were assessed in advanced gastric cancer. The authors observed that VEGFR-3 and CEA could help predict survival in advanced gastric cancer patients treated with oxaliplatin plus 5-fluorouracil and folinic acid (FOLFOX).

Innovations and breakthroughs

High serum VEGFR-3 levels are correlated significantly with poorer survival. In patients with a higher serum VEGFR-3 level, alternative chemotherapy regimens should be considered.

Applications

The results of this study will help predict survival in advanced gastric cancer patients treated with FOLFOX chemotherapy.

Peer review

In this paper, the authors analyzed the clinicopathological parameters, serum CEA, serum VEGFR-3 levels and survival of advanced gastric cancer patients treated with FOLFOX. Based on the results of the study, the authors concluded that a high serum VEGFR-3 level is significantly correlated to poorer survival of the patients.

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BRIEF ARTICLE

Peroxisome proliferator-activated receptor-γ 34C>G polymorphism and colorectal cancer risk: A meta-analysis

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Abstract

AIM: To investigate the association between peroxisome proliferator-activated receptor-γ (*PPAR-γ*) gene polymorphism 34 C>G and colorectal cancer (CRC), a meta-analysis review was performed in this report.

METHODS: A systematic literature search and selection of eligible relevant studies were carried out. Nine independent studies with a total number of 4533 cases and 6483 controls were included in the meta-analysis on the association between polymorphism 34 C>G and CRC.

RESULTS: There was no evidence for the association between PPAR-γ 34 C>G and CRC if all of the subjects in the nine studies were included. However, CG + GG showed a marginally significant difference from CC (OR = 0.84, 95% CI: 0.69-1.01, *P* = 0.07) in random-effect model. Stratified meta-analysis indicated that PPAR-γ 34 C>G was associated with colon cancer (OR = 0.8, 95% CI: 0.65-0.99, *P* = 0.04) in random-effect model, and the G allele decreased colon cancer risk. No significant association was observed between PPAR-γ 34 C>G and rectal cancer.

CONCLUSION: PPAR-γ 34 C>G is associated with colon cancer risk, but not associated with CRC and rectal cancer risk.

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Key words: Peroxisome proliferator-activated receptor-γ; Colorectal cancer; Polymorphism; Meta-analysis

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INTRODUCTION

Colorectal cancer (CRC) is one of the major causes of cancer death in developed countries, with over 9500 new cases in Netherlands in 2002 alone, for instance[1]. In 2003, it was estimated that about 147 500 new cancer cases and 57 100 deaths were caused by CRC in the USA[2]. With more than 71 000 new occurrences per year, the incidence and mortality rate of CRC in Germany is almost the highest all over the world[3]. Epidemiological and experimental evidences attributed CRC to both genetic and experimental factors were involved. Accumulating evidences suggested that the peroxisome

proliferator-activated receptor-γ (*PPAR-γ*) gene is related to CRC, which has been implicated in the pathogenesis of CRC in animal models and clinical studies. PPAR-γ is a member of the nuclear hormone receptor super-family, and plays a pivotal role in regulating adipocyte differentiation, glucose and lipid metabolism, insulin sensitivity, atherogenesis and immune[4]. A proline to alanine substitution has been detected in the *PPAR-γ* gene which is a common structural polymorphism (34 C>G, rs1801282) located at codon 12 (Pro12Ala) of PPAR-γ2-specific exon B. Although many studies were performed to investigate the relationship between the polymorphism 34 C>G of *PPAR-γ* gene and CRC, results were contradictory. Our study aims to confirm the former data of the association between *PPAR-γ* gene 34 C>G and CRC.

MATERIALS AND METHODS

Identification and eligibility of relevant studies

A systematic literature search in PubMed and Google was carried out in January 2010 using '*PPAR* gene', 'association' and 'CRC' with restriction to 'human' or 'homo sapiens'. Additional articles were identified through references cited in retrieved articles. Publications containing the same or overlapping data from the same authors were excluded. Studies were considered as eligible for the meta-analysis if the frequency of relevant genotypes was reported in both CRC cases and CRC-free controls, or in both colon cancer cases and CRC-free controls, as well as in both rectal cancer cases and CRC-free controls. Moreover, all of them were case-control studies or cohort studies. Nine articles reported on the analysis of the association between PPAR-γ Pro12Ala and CRC[1,2,4-10], four of which focused on the association between the polymorphism PPAR-γ Pro12Ala and colon cancer risk or rectal cancer risk[4-7].

Data extraction and statistical analysis

For each study, information was gathered about the first author, year of publication, country where the study was conducted and the distribution of each PPAR-γ 34 C>G genotype in cases and controls (Table 1). Some calculated data collected from the original data of the articles were applied in the subsequent meta-analysis.

Percentage of GG genotype in controls of each study was calculated, followed by Hardy-Weinberg Equilibrium (HWE) test in controls to determine the reliability of data, using a Chi-squared Goodness-of-fit Test by SPSS 13.0. Analysis was also conducted on inter-ethnicity difference in minor allele frequency. One-way ANOVA was used to compare more than two independent groups, while two-tailed *t* test was used to compare two independent groups by SPSS 13.0 software.

To investigate the effect of each allele, the ORs of G allele were calculated, referenced by C. Subsequently, pairwised combinations of genotypes were used to determine the hereditary models, including GG *vs* CC, CG *vs* CC, and GG *vs* CG, CG *vs* CC + GG, GG *vs* CC + CG and CG *vs* GG + GC, and the later genotype was used as a reference in each pair.

We also conducted meta-analyses for a combination of CG and GG genotypes *vs* CC genotype in each subgroups (European and USA population). In addition, stratified analyses were performed based on the case collection, including meta-analyses on the association between PPAR-γ 34 C>G and colon cancer risk and rectal cancer risk.

Heterogeneity among studies was tested to estimate which effect model, the fixed-effect one or the random-effect one, should be used. With a $P > 0.05$, the included studies were considered homogeneous and the fixed-effect model should be selected, otherwise, random-effect model should be used.

All of the meta-analyses above were conducted using Review Manager 4.2 software. The two-sided $P < 0.05$ was considered statistically significant.

RESULTS

Nine studies published from 2003-2007 were about the analysis on the relationship between PPAR-γ 34 C>G polymorphism and CRC risk, with a total number of 4533 cases and 6483 controls (Table 1). Seven studies (3870 cases/5028 controls) were conducted in Western countries, including 4 in Europe[1,5,9,10] and 3 in the USA[2,6,8]. Another two studies were performed in Asian countries[4,7]. There were four studies concerning colon cancer or rectal cancer with a total number of 2073 cases/3735 controls and 1321 cases/2765 controls, respectively[4-7].

The genotype distribution in the control groups in each study did not depart from the HWE with $P > 0.05$, except for two studies, in which HWE test could not be performed because of the incomplete data (Table 1).

The G allele frequency of PPAR-γ 34 C>G was 0.13 in the control group (626 cases/4694 controls) and 0.12 in the case group (424 cases/3600 controls), respectively. No statistical significance was found between case group and control group ($P = 0.381$). The G allele frequency of PPAR-γ 34 C>G in the Western controls (seven studies) was 0.14, the same as in the European controls (four studies). And in USA controls of three studies, G allele frequency of PPAR-γ 34 C>G was 0.13. In conclusion, there is no inter-ethnicity difference in minor allele frequency ($P = 0.968$).

Overall and subgroup-specific summary ORs and 95% CIs for the relationship between PPAR-γ 34 C>G and CRC risk are summarized in Table 2. For G *vs* C allele, GG *vs* CC, CG *vs* CC, GG *vs* CG, CG *vs* CC + GG, GG *vs* CC + CG and CG + GG *vs* CC genotypes of the overall study population, the fixed-effect and the random-effect ORs (95% CIs) were listed, respectively. Test for heterogeneity indicated that studies in the analyses for G *vs* C allele, CG *vs* CC, CG *vs* CC + GG, CG + GG *vs* CC genotypes, respectively were heterogeneous with $P < 0.05$. Hence, random-effect models were selected. And

Table 1 Study characteristics

No.	Ref.	Yr	Country	Number of cases					Number of controls					34G allele frequency in control	P^1
				CC	CG	GG	Total	CG + GG	CC	CG	GG	Total	CG + GG		
1	Landi *et al*[5]	2003	Spain (W, E)	311	46	3	360	49	243	61	5	309	66	0.11	0.773
2	Gong *et al*[2]	2005	USA (W)	129	30	4	163	34	153	52	7	212	59	0.16	0.743
3	Murtaugh *et al*[6]	2005	USA (W)	1840	-	-	2371	531	2283	-	-	2972	689	-	-
4	Jiang *et al*[4]	2005	India (A)	240	57	4	301	61	230	57	4	291	61	0.11	1.000
5	Siezen *et al*[1]	2006	Netherlands (W, E)	160	40	1	201	41	325	71	2	398	73	0.09	0.783
6	Koh *et al*[7]	2006	Singapore (A)	345	-	-	362	17	1075	-	-	1164	89	-	-
7	Gunter *et al*[8]	2006	USA (W)	153	41	4	198	45	146	37	1	184	38	0.11	0.838
8	Theodoropoulos *et al*[9]	2006	Greek (W, E)	164	48	10	222	58	118	70	12	200	82	0.24	0.950
9	Vogel *et al*[10]	2007	Denmark (W, E)	252	96	7	355	103	550	190	13	753	203	0.14	0.816

[1]*P* value for Hardy-Weinberg equilibrium (HWE) for peroxisome proliferator-activated receptor-γ (PPAR-γ) 34C>G polymorphism among controls. W: Western country; E: European country; A: Asian country; -: No data was shown in reference.

Table 2 Overall and group-specific summary statistics for PPAR-γ 34 C>G and colorectal, colon and rectal cancers

	No. of studies	Polymorphisms	P^1	Fixed-effect OR (95% CI), P^2	Random-effect OR (95% CI), P^3
Colorectal cancer					
Total	7	G *vs* C	0.010	0.88 (0.77, 1.00), 0.060	0.86 (0.69, 1.08), 0.210
	7	GG *vs* CC	0.720	0.85 (0.53, 1.35), 0.480	0.83 (0.52, 1.33), 0.440
	7	CG *vs* CC	0.020	0.86 (0.74, 1.00), 0.050	0.83 (0.65, 1.07), 0.160
	7	GG *vs* CG	0.990	1.10 (0.68, 1.79), 0.690	1.09 (0.67, 1.78), 0.720
	7	CG *vs* CC + GG	0.020	0.86 (0.74, 1.00), 0.060	0.84 (0.66, 1.07), 0.150
	7	GG *vs* CC + CG	0.830	0.91 (0.57, 1.44), 0.680	0.89 (0.56, 1.43), 0.630
	9	CG + GG *vs* CC	0.009	0.90 (0.82, 0.99), 0.030	0.84 (0.69, 1.01), 0.070
Western	6	G *vs* C	0.006	0.87 (0.75, 1.00), 0.050	0.85 (0.65, 1.11), 0.230
	6	GG *vs* CC	0.600	0.83 (0.51, 1.36), 0.470	0.81 (0.49, 1.34), 0.420
	6	CG *vs* CC	0.009	0.84 (0.72, 1.00), 0.040	0.81 (0.60, 1.09), 0.170
	6	GG *vs* CG	0.970	1.12 (0.67, 1.87), 0.670	1.11 (0.66, 1.86), 0.700
	6	CG *vs* CC + GG	0.010	0.85 (0.72, 1.00), 0.050	0.82 (0.61, 1.09), 0.160
	6	GG *vs* CC + CG	0.730	0.90 (0.55, 1.47), 0.680	0.88 (0.54, 1.45), 0.630
	7	CG + GG *vs* CC	0.006	0.91 (0.82, 1.01), 0.070	0.85 (0.68, 1.06), 0.140
USA	3	CG + GG *vs* CC	0.320	0.95 (0.84, 1.07), 0.360	0.94 (0.80, 1.11), 0.450
Europe	4	CG + GG *vs* CC	0.002	0.84 (0.70, 1.00), 0.060	0.79 (0.52, 1.19), 0.260
Colon cancer	4	CG + GG *vs* CC	0.030	0.83 (0.72, 0.96), 0.010	0.80 (0.65, 0.99), 0.040
Rectal cancer	4	CG + GG *vs* CC	0.050	0.98 (0.82, 1.18), 0.860	0.84 (0.58, 1.22), 0.370

[1]*P* value for test for heterogeneity; [2]*P* value for fixed-effect model; [3]*P* value for random-effect mode.

for the rest ones with $P > 0.05$, fixed-effect model was used. As a result, no statistical significance was observed among the above analyses. However, the data of CG + GG *vs* CC genotypes was marginally significant with OR = 0.84 (95% CI: 0.69-1.01, $P = 0.07$).

Limited results in studies from Western countries, the fixed-effect and random-effect ORs (95% CIs) for G *vs* C allele, GG *vs* CC, CG *vs* CC, GG *vs* CG, CG *vs* CC + GG, GG *vs* CC + CG and CG + GG *vs* CC genotypes are listed in Table 2, respectively. Same to the former analyses of the total study population, there was no evidence for the association between PPAR-γ 34 C>G and CRC risk.

Further subgroup-specific analyses were performed in the European and USA studies. In studies from European countries, summary ORs (95% CIs) for CG + GG *vs* CC genotypes in fixed-effect model and random-effect model were 0.84 (0.70, 1.00) and 0.94 (0.80, 1.11), respectively. In studies from the USA, the corresponding ORs (95% CIs) were 0.79 (0.52, 1.19) and 0.95 (0.84, 1.07), respectively. No evidence was found for the association between PPAR-γ 34 C>G and CRC risk in each of the two study populations.

However, when stratified analyses were performed, the results were different. As shown in Table 2, four studies were involved in the meta-analyses for the association of PPAR-γ 34 C>G with colon cancer risk and rectal cancer risk. In the four studies, only the data for CG + GG *vs* CC genotypes was sufficient enough for the analyses. As *P* values of the heterogeneity test in the two meta-analyses were less than 0.05, random-effect model was used. Summary ORs (95% CIs) for CG + GG *vs* CC genotypes in colon cancer studies and rectal cancer studies were 0.80 (0.65, 0.99) and 0.84 (0.58, 1.22),

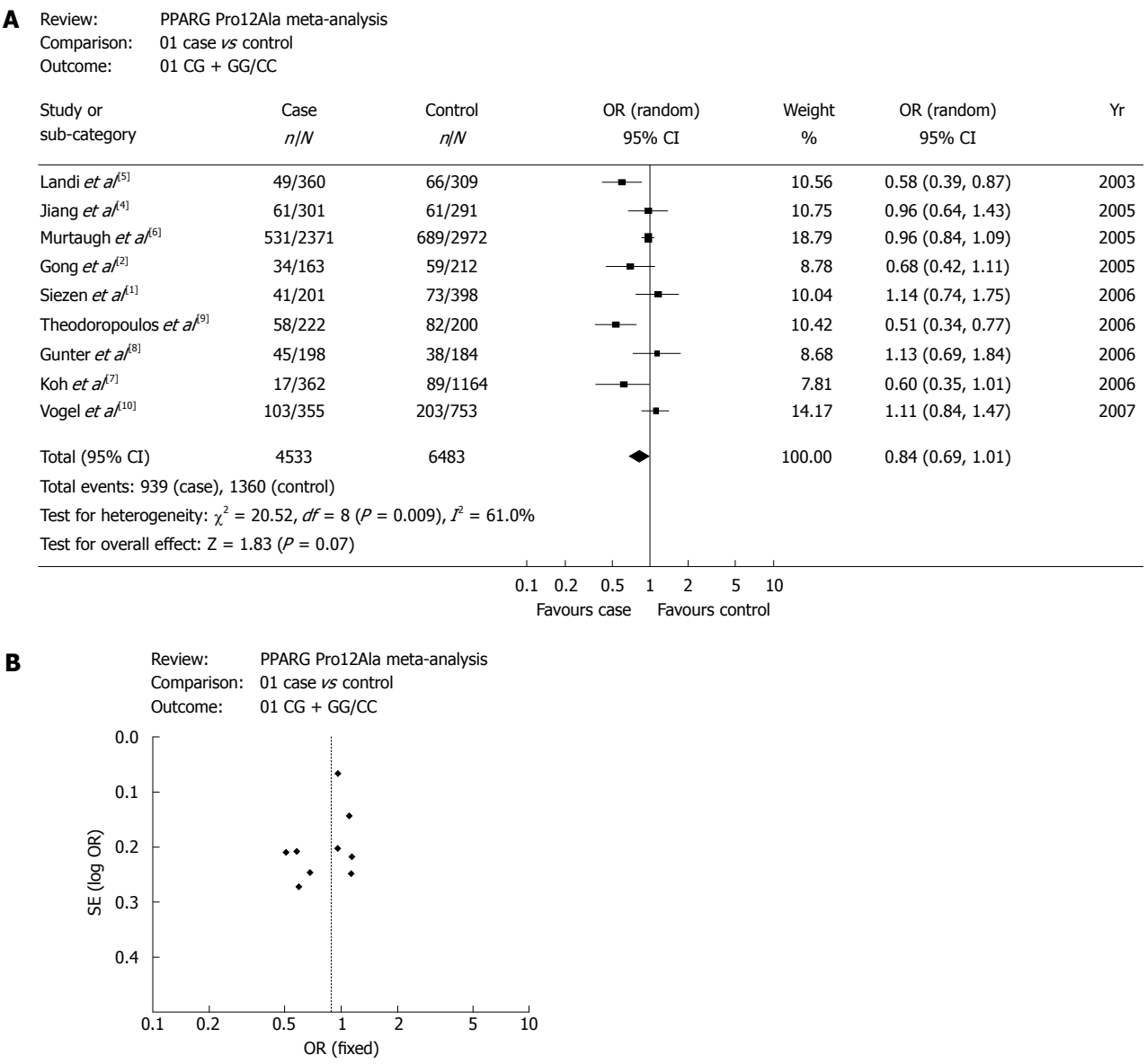


Figure 1 Association between peroxisome proliferator-activated receptor-γ (PPAR-γ) 34 C>G and colorectal cancer risk (CG + GG *vs* CC). A: Forest plot-studies are sorted in order of publication year; B: Funnel plots for the associations.

respectively. Statistical significance was observed in the association meta-analysis between PPAR-γ 34 C>G and colon cancer risk (P = 0.04), indicating that PPAR-γ 34 C>G was associated with colon cancer risk, and G allele decreased the colon cancer risk. However, there was no evidence for the association between PPAR-γ 34 C>G and rectal cancer risk (P = 0.37).

Figure 1A shows the forest plots of meta-analysis for CG + GG *vs* CC genotypes to confirm the association between PPAR-γ 34 C>G and CRC risk in the overall study population. The association between PPAR-γ 34 C>G and CRC risk had a marginally statistical significance. Figure 1B is the funnel plot, suggesting that there was no publication bias in the studies.

Figure 2A displays the forest plots of the study on the association between PPAR-γ 34 C>G and colon cancer risk. Obviously, there was a significant association of PPAR-γ 34 C>G with colon cancer risk. Publication bias was not found in this study as shown in Figure 2B. Other forest plots and funnel plots of meta-analyses on the association between PPAR-γ 34 C>G and CRC risk, colon cancer risk and rectal cancer risk were not shown. However, the results are presented in Table 2.

DISCUSSION

CRC is one of the leading causes of cancer death in the developed countries[11,12]. Both sporadic and hereditary CRC is caused by a set of molecular events[13]. Accumulated evidences indicate that lipid metabolism, especially the one involved in the arachidonic acid (AA)-pathway, appears to play a critical role in the development of colorectal tumor[14]. *PPARγ* gene, one of the most important components of the AA-pathway, has been veri-

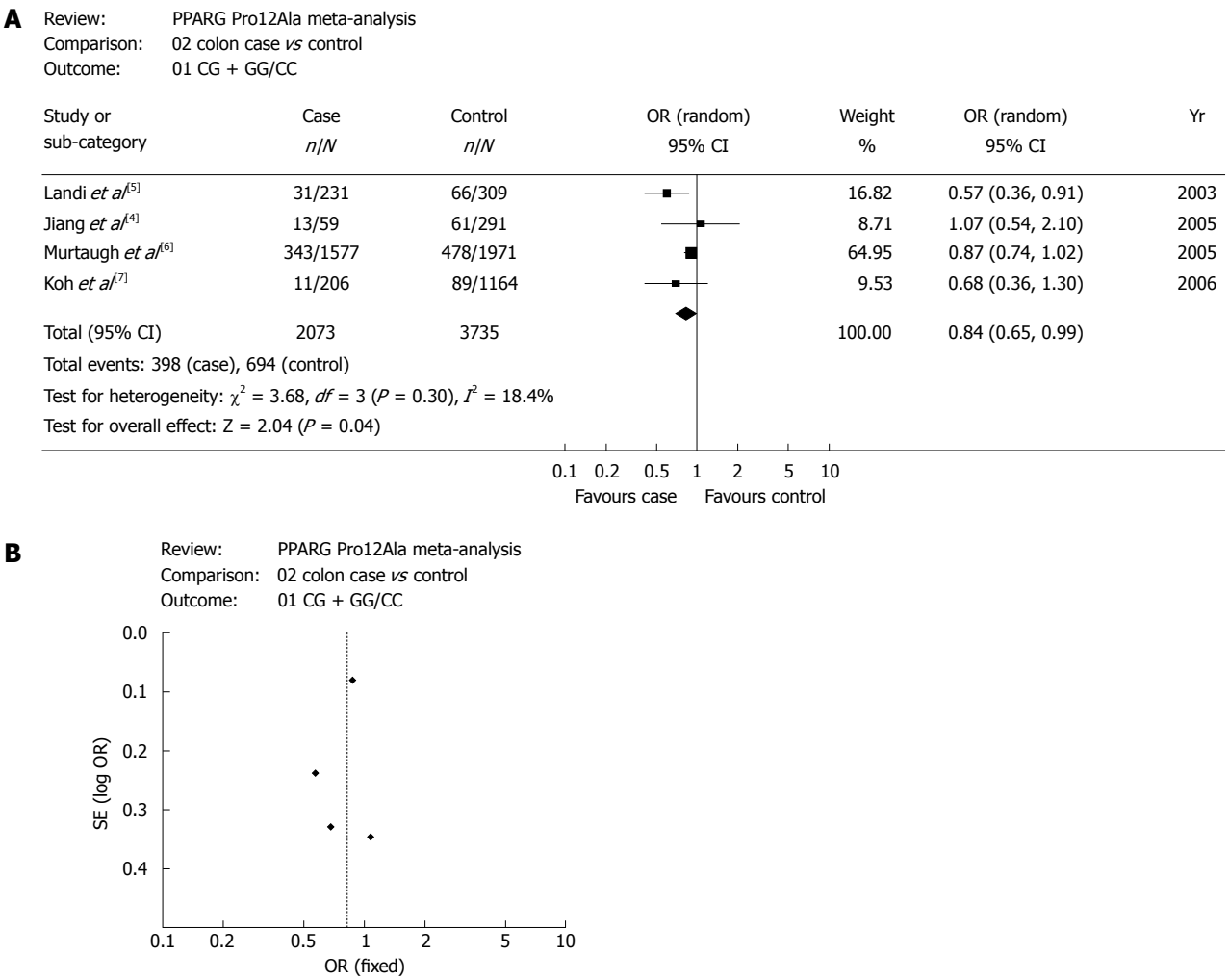


Figure 2 Association between PPAR-γ 34 C>G and colon cancer risk (CG + GG *vs* CC). A: Forest plot-studies are sorted in order of publication year; B: Funnel plots for the associations.

fied to express in a variety of tumor cells. And it will lead to either inhibition of cell proliferation or induction of apoptosis after bonding with ligands[15,16]. Many studies reported that PPAR-γ was also expressed in colon tumors, normal colon mucosa and colon cancer cell lines[17-19]. Genomics research showed that there was a polymorphism in the coding region 34 C>G in PPAR-γ which resulted in the amino acid change of Pro12 Ala[20]. So far, many studies have been performed on the association between PPAR-γ 34 C>G polymorphism and CRC risk, but produced controversial results.

According to our search of references, no systematic review has been published on the analysis of the association between PPAR-γ 34 C>G and CRC. In order to confirm the data on the associations between *PPAR-γ* gene polymorphism and CRC, we did a meta-analysis based on nine studies from Europe, Asia and the USA.

Our results showed that there was no evidence for the association between PPAR-γ 34C>G and CRC if all of the subjects in the nine studies were included. Subgroup-specific meta-analyses also indicated that there was no association between PPAR-γ 34C>G and CRC in European, Asian and the USA studies. As shown in our analysis, G allele might decrease CRC risk, although statistical difference was not significant in these meta-analyses.

Many studies have indicated that both genetic and environmental factors are involved in the development of colorectal tumor[21]. Environmental factors include ethnicity, gender, diet, age, NSAIDS use, BMI, smoking, drinking, family history of disease and so on. It was inferred that there must be interaction between the environmental factors and *PPAR-γ* gene, which had been proved in many researches, including the nine studies. Therefore, interaction is one of the factors of the meta-analyses. Further analysis should be conducted to confirm the influence of PPAR-γ 34 C>G on CRC risk.

Stratified meta-analysis indicated that PPAR-γ 34 C>G was associated with colon cancer, and the G allele decreased the colon cancer risk. No evidence was observed for the association between PPAR-γ 34 C>G and rectal cancer.

In conclusion, no evidence was observed for the association between PPAR-γ 34 C>G and CRC risk and rectal cancer risk. However, PPAR-γ 34 C>G is associated with colon cancer risk, which is meaningful to early

diagnosis, prevention and individual-based treatment of colon cancer. Furthermore, 34 C>G of *PPAR-γ* gene might be a potential therapeutic target for colon cancer.

COMMENTS

Background

Colorectal cancer (CRC) is one of the major causes of cancer death in the developed countries. Accumulated evidences suggest that the peroxisome proliferator-activated receptor-γ (*PPAR-γ*) gene is related to CRC.

Research frontiers

Accumulated evidences indicate that lipid metabolism, especially the one in the arachidonic acid (AA)-pathway, appears to play a critical role in the development of CRC. *PPAR-γ* gene, one of the most important components of the AA-pathway, has been verified to express in a variety of tumor cells. A proline to alanine substitution in the *PPAR-γ* gene was detected, which might be associated with CRC.

Innovations and breakthroughs

Many studies have been performed about the association between the polymorphism 34 C>G of *PPAR-γ* gene and CRC, but got conflicting results. In order to confirm the data, meta-analyses, as a better statistical analysis technique, were performed in this report.

Applications

In this report, the association between *PPAR-γ* gene polymorphism 34 C>G and colon cancer risk was observed, and the G allele decreased the colon cancer risk, which is meaningful to early diagnosis, prevention and individual-based treatment of colon cancer. Furthermore, 34 C>G of *PPAR-γ* gene might be a potential therapeutic target for colon cancer.

Terminology

Susceptibility to colon cancer differs according to genotype of PPAR-γ 34 C>G, and people with G allele might have a lower colon cancer risk.

Peer review

This is an interesting meta-analysis on the association between PPAR-γ 34C>G polymorphism and CRC risk. The main finding of the paper was that PPAR-γ 34 C>G was weakly associated with colon cancer risk, but was not associated with CRC and rectal cancer risk. The methods are properly used; authors report a high quality meta-analysis.

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BRIEF ARTICLE

Aberrant expression of nuclear matrix proteins during HMBA-induced differentiation of gastric cancer cells

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Abstract

AIM: To investigate the aberrant expression of nuclear matrix proteins in human gastric cancer cells before and after hexamethylene bisacetamide (HMBA) treatment.

METHODS: Proteomics analysis of differential nuclear matrix proteins was performed by two dimensional electrophoresis polyacrylamide gel electrophoresis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The expression levels of three nuclear matrix proteins were further confirmed by Western blotting and their locations in nuclear matrix filament were observed by quantum dots-based immunofluorescence.

RESULTS: Proteomics analysis showed that 43 protein spots were significantly changed due to HMBA treatment. Fifteen proteins were identified in the HMBA-induced differentiation of gastric tumor cells. Eight proteins spots were down-regulated while seven were up-regulated. Among these proteins, prohibitin, nucleophosmin and hnRNP A2/B1 were significantly decreased in HMBA-treated human gastric cancer cells, and their locations in nuclear matrix were altered by HMBA. Our results proved the alteration of specific nuclear matrix proteins during the differentiation of human gastric cancer cells. And the aberrant expressions of nuclear matrix proteins were of significance in revealing the regulatory mechanism of tumor cell proliferation and differentiation.

CONCLUSION: The aberrant expressions and intracellular redistributions of nuclear matrix proteins before and after HMBA treatment indicated that nuclear matrix proteins play a pivotal role in the differentiation of gastric cancer cells.

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Key words: Human gastric tumor cell; Hexamethylene bisacetamide; Differentiation; Nuclear matrix; Proliferation

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INTRODUCTION

Gastric cancer is the most common malignant gastrointestinal cancer, which accounts for 25% of cancer

deaths[1]. Researches on gastric cancer cell differentiation will not only contribute to the diagnosis of gastric cancer, but also the regulatory mechanism of tumor cell differentiation. Studies have been carried out on the mechanism of induced differentiation of gastric cancer cells. But reports about the proteins related to the differentiation and proliferation of gastric cancer are rare. It is important to elucidate the regulatory mechanism of gastric cancer cell differentiation. Nuclear matrix is a filamentous protein framework for eukaryotic cell chromatin. Nuclear matrix proteins closely relate to DNA duplication and transcription[2]. Some regulatory proteins or enzymes are nuclear matrix proteins or nuclear matrix-binding proteins[3], and they regulate cell differentiation. Protein components of nuclear matrix are dynamic throughout cell proliferation and differentiation. Protein composition of nuclear matrix in tumor cells differ from normal tissue-derived cells[4]. In our previous study we found that the protein compositions of nuclear matrix were changed during the induced-differentiation of various tumor cells[5,6], suggesting that analysis on the aberrant expression of nuclear matrix proteins was of great importance for an in-depth investigation in the mechanism of cell carcinogenesis and reversal. Hexamethylene bisacetamide (HMBA) is a hybrid bipolar compound and originally developed as a potent inducer of cell differentiation[7]. Hereby, based on the induced effects of HMBA on the differentiation of human gastric cancer cells[8,9], we extended our study to determine the aberrant expression of nuclear matrix proteins in HMBA-treated human gastric adenocarcinoma BGC-823 cells. We aimed to identify specific nuclear matrix proteins related to gastric cancer cells and provide further scientific evidences for the mechanism of gastric cancer cell proliferation and differentiation.

MATERIALS AND METHODS

Cell culture

BGC-823 cells, obtained from China Center for Type Culture Collection, were routinely cultured in RPMI 1640 (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone) and 100 U/mL penicillin, 100 μg/mL streptomycin and 50 μg/mL kanamycin at 37℃ in a humidified atmosphere containing 5% CO_2.

Whole cell and nuclear matrix lysates preparation

To prepare the whole cell extracts, cells were washed in phosphate buffered saline (PBS) and then lysed in ice-cold lysis buffer {7 mol/L urea, 2 mol/L thiourea, 4% 3-[(3-Cholanidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 1.5% Triton X-100, 1% pharmalyte (pH 3-10 Bio-rad), 65 mmol/L DL-Dithiothreitol (DTT), 40 mmol/L Tris, 5 mg/mL aprotinin, 1 mg/mL leupeptin, 1 mg/mL pepstatin, 2 mmol/L phenylmethanesulfonyl fluoride (PMSF), and 5 mmol/L ethylenediaminetetraacetic acid (EDTA)}. The suspension was then sonicated for 20 min at 0℃ and centrifuged at 8000 × *g* for 30 min.

Nuclear matrix proteins were prepared using a method described by Michishita *et al*[10]. After washed in ice-cold PBS twice, BGC-823 cells were suspended in cytoskeleton (CSK) buffer [100 mmol/L KCl, 3 mmol/L $MgCl_2$, 5 mmol/L ethylene glycol tetraacetic acid, 10 mmol/L piperazine-N,N'-bis(2-ethanesulfonic acid), 300 mmol/L sucrose, 0.5% Triton X-100, and 2 mmol/L PMSF, pH 6.8] for 10 min at 0℃. After being centrifuged at 1000 × *g* for 5 min, the pellet was resuspended in digestion buffer (identical to CSK buffer except for 50 mmol/L NaCl instead of KCl) containing 400 mg/mL DNase I for 30 min at room temperature and centrifuged at 1000 × *g* twice. Cold ammonium sulfate at a final concentration of 0.25 mol/L was used to precipitate proteins. After centrifugation, the pellet was dissolved in lysis buffer [7 mol/L urea, 2 mol/L thiourea, 4% CHAPS, 1.5% Triton X-100, 1% Pharmalyte (pH 3-10 Bio-rad), 65 mmol/L DTT, 40 mmol/L Tris, 5 mg/mL aprotinin, 1 mg/mL leupeptin, 1 mg/mL pepstatin, 2 mmol/L PMSF, and 5 mmol/L EDTA] and then sonicated at 0℃ for 20 min. Finally, the suspension was centrifuged at 10000 × *g* at 4℃ for 30 min and the supernatants were used as nuclear matrix extracts.

Protein concentrations were determined by BCA assay.

Two dimensional electrophoresis, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis and protein identification

2-D polyacrylamide gel electrophoresis (PAGE) was performed as follows. Protein lysates were diluted in sample buffer with 2% Immobiline™ DryStrip gel (IPG) buffer, pH 3-10, nonlinear (GE Heathcare). The samples were applied to 18 cm, immobilized pH gradient strips of pH 3-10 (IPG Drystrips, GE Healthcare). After isoelectric focusing was completed, the strips were equilibrated and the second dimensional electrophoresis was carried out overnight at 3 W/gel at 20℃. The triplicate sets of silver-stained gels were scanned using a UMAX POWER LOOK Ⅲ photometer and analyzed with the PD Quest 8.0 software (Bio-rad). The digitalized two dimensional electrophoresis (2-DE) gel images were compared by matching method. Differentially expressed spots were analyzed and annotated.

The spots were cut and digested using 12.5 ng/μL trypsin (Promega, Madison, WI, USA) in 50 mmol/L ammounium bicarbonate (pH 8.0, Sigma). After that the samples were eluted with 2 μL of matrix solution containing 10 mg/mL α cyano-4-hydroxy cinnamic acid (CHCA, Sigma) and were submitted to Bruker Ⅲ matrix-assisted laser desorption/ionization time-of-flight mass spectrometer. The spectra were internally calibrated using the trypsin autolysis products [842.51 (M + H) and 2211.11 (M + H)] by Flex Analysis software and searched against Swiss-prot and NCBI database using the Mascot tool from matrix science. All the searches were analyzed with a 50 ppm mass tolerance.

Western blotting

For Western blotting experiments, 20 μg cell lysates were

Table 1 Differentially expressed nuclear matrix proteins identified by mass spectrum

Spot number	Protein name	Accession number	Mol. mass calc (Da)	pI (calc)	Score
Up-regulated proteins					
L1	Zinc finger protein 268	Q14587	108373	9.14	57
L2	Calcium-binding protein CaBP5	Q9NP86	19812	4.46	61
L3	MAGUK p55 subfamily member 3	Q13368	66168	6.27	45
L4	Vacuolar protein sorting 33B	Q9H267	70570	6.29	54
L5	Ras-related protein Rab-30	Q15771	23058	4.91	64
L6	Vimentin	57471646	49623	5.19	48
L7	Calnexin (Precursor)	4262127	10638	5.30	89
Down-regulated protein					
L8	Peroxiredoxin 2	P32119	21892	5.66	72
L9	Transcriptional repressor CTCFL	Q8NI51	75668	8.58	67
L10	Prohibitin	P35232	29859	5.57	79
L11	Keratin 1, type Ⅱ, cytoskeletal	7428712	65455	6.03	86
L12	PACAP protein	18204192	20681	5.37	161
L13	hnRNP A2/B1	P22626	36600	8.67	97
L14	C-type lectin	Q2K157	22130	5.47	68
L15	Nucleophosmin	Q96EA5	32726	4.64	76

loaded and separated on polyacrylamide gles and then transferred to positively-charged nylon membranes (Millipore, Bedford, MA) according to standard protocol. These blots were blocked for 1 h at room temperature in 5% skim milk. The target proteins were probed with primary antibodies and horseradish peroxidase-labeled secondary antibodies (Santa Cruz). β-actin was used as an indicator for equality of lane loading. Antibody positive bands were visualized using ECL Western blotting detection reagents (Pierce). The X-ray film was scanned and the band density was calculated using the ImageJ software[11].

Quantum dots-based sample preparation for fluorescence microscopy

The NM-IF system was prepared according the methods described by Liang *et al*[12]. After being prefixed with 4% paraformaldehyde at room temperature for 10 min, the cover slips were blocked with 3% bovine serum albumin for 1 h and then incubated with nucleophosmin (NPM), prohibitin (PHB) and hnRNP A2/B1 primary antibodies at 37℃ for 1 h. After washing, the cover slips were rinsed in biotin-labeled secondary antibodies for 45 min at 37℃, washed with tris-buffered saline Tween-20, and incubated with streptavidin-conjugated quantum dots (size scale, 605 nm) for 1 h at 37℃. After that, the cover slips were enveloped with 90% glycerol and observed under fluorescence microscope.

RESULTS

Proteomics analysis of BGC-823 cells before and after HMBA treatment

For proteomics analysis, cell lysates from control and HMBA-treated cells were submitted to 2D-PAGE followed by silver-staining of the gel. The procedures were independently repeated three times and the representative gel images are shown in Figure 1A. And Figure 1B displayed the enlarged maps of changed expression of nuclear matrix proteins from BGC-823 cells. PD Quest 8.0 software (Bio-rad) detected about 342 ± 10 proteins spots on the silver-stained gels of control group while 280 ± 13 protein spots of HMBA-treated group, suggesting that the result of 2-DE PAGE had a high repeatability. Forty-three changed spots were excised and digested with trypsin, and were identified by mass spectrometry. Fifteen proteins were identified in the HMBA-induced differentiation of gastric tumor cells. The identified proteins are listed in Table 1. The expressions of Zinc finger protein 268, calcium-binding protein CaBP5, MAGUK p55 subfamily member 3, vacuolar protein sorting 33B, and Ras-related protein Rab-30, Vimentin, Calnexin (Precursor) were up-regulated while peroxiredoxin 2, transcriptional repressor CTCFL, PHB, Keratin 1 type Ⅱ cytoskeletal, PACAP protein, hnRNP A2/B1, C-type lectin and NPM were down-regulated. Another 28 protein spots were not identified for their low abundances. We next focused on PHB, NPM and hnRNP A2/B1, which were also identified as aberrantly expressed proteins in our previously studies[12-15].

Immunoblotting confirmation of differentially expressed nuclear matrix proteins

To further verify the aberrant changes of PHB, NPM and hnRNP A2/B1, Western immunoblotting was employed to confirm the expression levels of these proteins in cells before and after HMBA treatment, and the intensities of protein bands were densitometrically quantified as described by Sheffield JB[11]. Results showed that signals of PHB, NPM and hnRNP A2/B1 in HMBA-treated cells were much lower than that in control cells, suggesting that the expressions of these three proteins at 7 d of treatment were significantly inhibited by HMBA (Figure 2). All the Western blotting results were consistent with the proteomic analysis.

Localization of PHB, NPM and hnRNP A2/B1 in NM-IF system before and after HMBA treatment

Proteomics analysis showed that PHB, NPM and hnRNP

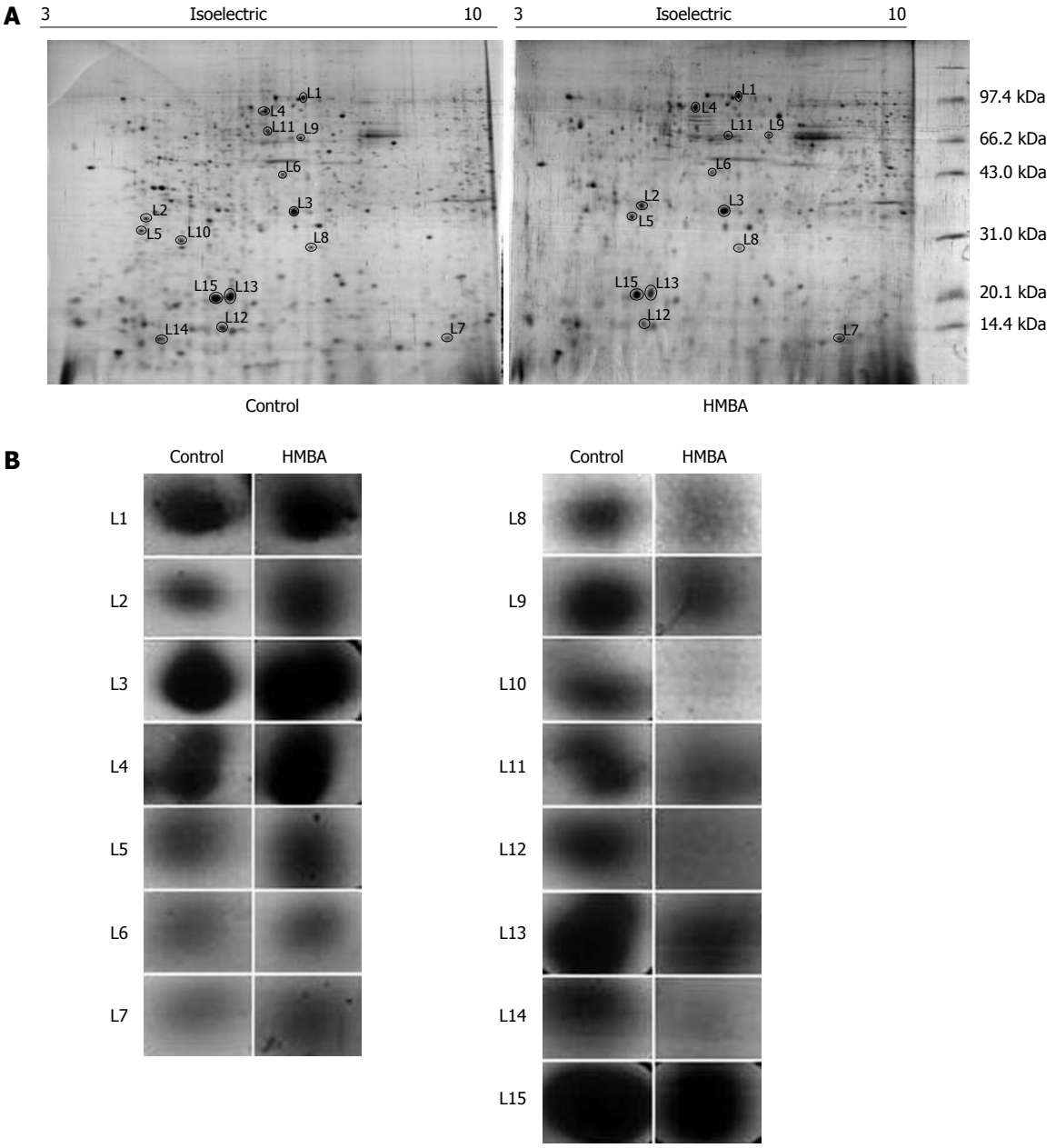


Figure 1 Two-dimensional protein profiles from the nuclear matrix of BGC-823 cells. A: Representative two-dimensional protein profiles from the nuclear matrix of BGC-823 cells before and after hexamethylene bisacetamide (HMBA) treatment. The differentially expressed proteins are marked with circular symbols on the gels. L1-L7 spots indicate the up-regulated proteins while L8-L15 indicates the down-regulated proteins; B: Enlarged portions from 2-DE gels.

A2/B1 were nuclear matrix proteins. QDs-based location experiments were conducted to corroborate the alteration of the physical distribution of these three proteins in nuclear matrix-intermediate system. Clearly altered distribution patterns for NPM and hnRNP A2/B1 were observed in the nucleus while PHB in at the cytoplasm. The red fluorescence representing PHB mainly accumulated at the nucleus periphery in control cells (Figure 3C), while PHB was uniformly distributed in the whole cytoplasm after HMBA treatment, suggesting that HMBA obviously altered the location of PHB (Figure 3D). Highly intensified NPM fluorescence mainly localized in the residue of nucleoli in correspondence with the fact that NPM was an established marker for the granular components[16], with very subtle expression in the cytoplasm (Figure 3A). HMBA treatment resulted in the low intensity of fluorescence in nucleolus and faint fluorescence in cytoplasm derived from NPM, suggesting that NPM had a tendency of translocation from nucleoli regions to nuclear matrix (Figure 3B). When referred to hnRNP A2/B1, a similar distribution pattern to NPM was observed. In control cells, the fluorescence representing hnRNP A2/B1 was mainly detected in nuclear region with subtle signals in cytoplasm region (Figure 3E), whereas after HMBA treatment, a clear translocation appeared from nuclear to cytoplasm region, and hnRNP A2/B1was uniformly distributed in the cytoplasm but not in the nucleus (Figure 3F).

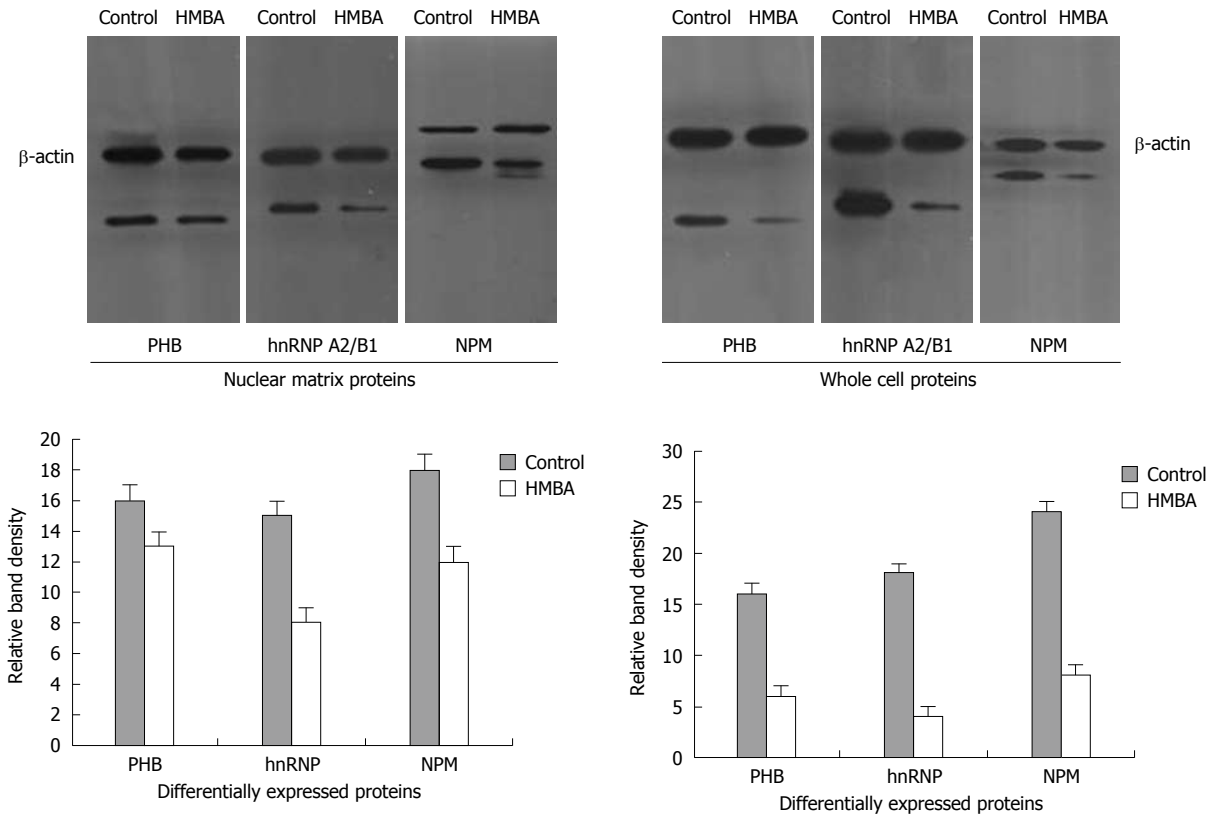


Figure 2 Determination of prohibitin (PHB), nucleophosmin (NPM) and hnRNP A2/B1 at nuclear matrix proteins and whole cell proteins by Western immunoblotting. All of these three protein signals were found to be down-regulated at whole cell level and nuclear matrix level after HMBA treatment.

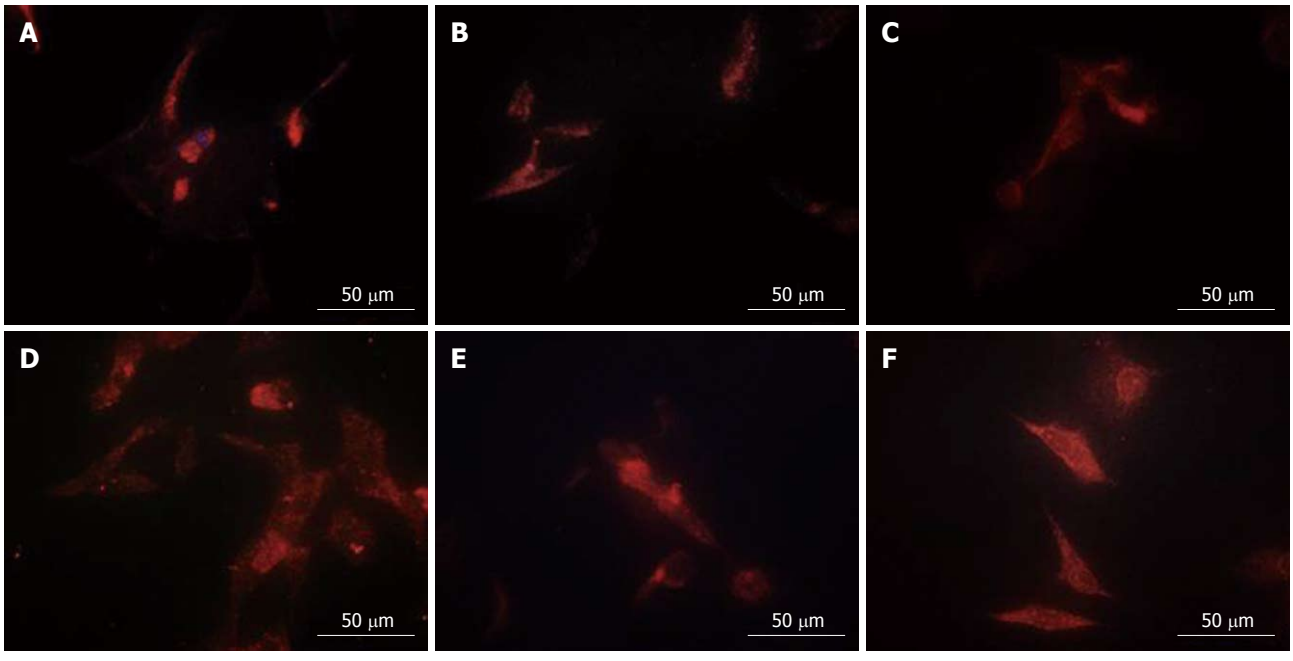


Figure 3 Effects of HMBA treatment on the localization of differentially expressed nuclear matrix proteins in NM-IF system as shown by a quantum dots-based immnuofluorescence. Typical changes of localization of NPM (A, B), PHB (C, D) and hnRNP A2/B1 (E, F) are observed after treatment with 5 mmol/L HMBA. The figure is composed of representative pictures taken from three independent experiments. A, C, E: Control; B, D, F: HMBA.

DISCUSSION

The alteration of nuclear matrix components is closely related to cell carcinogenesis. Configuration and protein composition of nuclear matrix in tumor cells differ from normal tissue-derived cells. For further investigating the effects of HMBA on the protein components of nuclear matrix of human gastric cancer cells, proteomics

analysis was employed to screen the aberrant nuclear matrix proteins of human gastric cancer cells before and after HMBA treatment. And 15 differentially expressed proteins were identified, seven of which, including Zinc finger protein 268, nebulin, *etc.* were up-regulated while the other eight proteins, including PHB, KeHMBAtin 1 type Ⅱ, cytoskeletal, hnRNP A2/B1, NPM, *etc.* were down-regulated. Getzenberg *et al*[17] reported that nuclear matrix profiles of bladder cancer, liver cancer and colon cancer were significantly altered in comparison with that of the normal tissues; moreover, differential nuclear matrix proteins were tumor-specific[17-19]. In our previous studies, we found variational nuclear matrix profiles of tumor cells[5,6]. HMBA could change protein compositions of gastric cancer cell nuclear matrix. The alteration of nuclear matrix protein expressions during the differentiation of gastric cancer cells was closely related to their functional transformation.

In this article we focused on the aberrant expression of nuclear matrix proteins in the induced-differentiation of gastric cancer cells. Among all the differentially expressed proteins, the down-regulated proteins, PHB, NPM and hnRNP A2/B1, were also identified in our previous studies on the induced differentiation of gastric cancer MGC80-3 cells, suggesting that PHB, NPM and hnRNP A2/B1 were common aberrantly expressed nuclear matrix-specific proteins in different tumor cell lines[8,12-15]. Proteomics analysis showed that expressions of PHB, NPM and hnRNP A2/B1 were down-regulated both in the whole cell lysates and nuclear matrix lysates, and the expression changes were further confirmed by Western blotting. Quantum dots-based immunofluorescent assay was performed to investigate the localization of specific nuclear matrix proteins in nuclear matrix-intermediate filament system. And results displayed that PHB, NPM and hnRNP A2/B1 were localized in nuclear matrix fibers, and their locations were altered by HMBA treatment. Our data further proved that PHB, NPM and hnRNP A2/B1 were common differential nuclear matrix proteins. And the aberrant expression and relocation of these three proteins were closely related to their regulatory roles in the process of tumor cell differentiation.

The importance of differentially expressed nuclear matrix proteins was embodied not only by their locations in nuclear matrix fibers but also their regulations on cell differentiation and apoptosis. For example, PHB presented anti-proliferation activities by inhibiting the activity of nuclear estrogen receptors, and it was usually over-expressed in tumor tissues[20]. PHB was also a biomarker protein for differentiating benign prostate hyperplasia from prostate cancer[21]. NPM was an abundant nucleolar phosphoprotein, and it could cause the suppression of cell differentiation and anti-apoptosis in human carcinogenesis. Inhibiting the activity of NPM led to the over-expression of P53[22]. Cell proliferation could be inhibited by blocking the shuttle of NPM from nuclear to cytoplasm[23]. hnRNP A2/B1 played a pivotal role in DNA damage repair[24]. The expression and location of PHB, NPM and hnRNP A2/B1 were changed in gastric tumor cells before and after HMBA treatment, suggesting their pivotal biological functions in the differentiation of gastric tumor cells. Continued investigation on specific nuclear matrix proteins will contribute to better understanding the regulatory mechanisms of nuclear matrix proteins in gastric carcinogenesis and reversal.

COMMENTS

Background

Gastric cancer is the most common malignant gastrointestinal cancer and accounts for 25% of cancer deaths. Nuclear matrix proteins are closely related to DNA duplication and transcription. Researches on nuclear matrix proteins of gastric tumor cells will contribute to the diagnosis and prevention of gastric cancer.

Research frontiers

It is important to identify specific nuclear matrix proteins related to gastric cancer cells and provide further scientific evidences for the mechanism of gastric cancer cell proliferation and differentiation.

Innovations and breakthroughs

Forty-three protein expressions were significantly changed in hexamethylene bisacetamide (HMBA)-treated human gastric cancer cells. Moreover, the locations of three proteins in nuclear matrix fibers were obviously altered after HMBA treatment.

Applications

The differentially expressed nuclear matrix proteins might be intracellular target proteins of HMBA and the tumor markers for gastric cancer.

Peer review

This study examined the effects of HMBA on nuclear matrix protein expression in gastric cancer cell BGC-823. Forty-three proteins were affected significantly by the treatment, among them 15 were identified including 7 up-regulated and 8 down-regulated ones. The study is neatly designed and the figures are presented with good quality. The manuscript is highly interesting, well-written and valuable.

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CASE REPORT

Refractory gastric ulcer with abundant IgG4-positive plasma cell infiltration: A case report

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Abstract

We describe a 77-year-old man with refractory gastric ulcer that worsened after *Helicobacter pylori* eradication therapy. Pathology showed marked infiltration of IgG4-positive plasma cells in the gastric lesions, which led us to suspect IgG4-related sclerosing disease. To the best of our knowledge, this is the first report of IgG4-related gastric ulcer without the main manifestation of autoimmune pancreatitis.

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Key words: Sclerosis; Gastric ulcer; IgG4; Plasma cells

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INTRODUCTION

Gastric ulcer is now recognized as an infectious disease that results from infection with *Helicobacter pylori* (*H. pylori*). *H. pylori* causes continuous gastric inflammation with abundant neutrophil infiltration, and eradication therapy is standard treatment in gastric ulcer[1]. We encountered a 77-year-old man with gastric ulcer of 5 years' duration, which worsened after *H. pylori* eradication therapy. Pathologically, gastric lesions show marked infiltration of plasma cells, and immunohistochemical study revealed frank IgG4-positive plasma cell infiltration. This clinical picture suggested refractory gastric ulcer that was suspected of being IgG4-related sclerosing disease.

CASE REPORT

A 72-year-old man visited the gastroenterology department of our hospital in June 2004 complaining of abdominal discomfort and appetite loss. He had been well until 1 mo earlier, when he experienced discomfort in the upper abdomen and decreased appetite. He had no abdominal pain, diarrhea, fevers, night sweats or body weight loss. He had no allergies, and was not taking any medication. He had undergone appendectomy at age 21 years. He had no history of tobacco smoking and drank alcohol socially. There was no family history of cancer or autoimmune disease. On examination, he appeared comfortable, with a temperature of 37.3℃, blood

pressure 110/70 mm Hg, and pulse 128 beats/min. The abdomen was soft, without tenderness or distension. The remainder of the examination was normal.

Esophagogastroduodenoscopy (EGD) revealed multiple ulcers in the stomach, while the rapid urease test (RUT) was negative (Figure 1A). Biopsy specimens revealed no malignant cells. Treatment with famotidine was initiated at a single daily dose of 20 mg, which resulted in the gradual resolution of symptoms. EGD at 2 mo revealed multiple gastric ulcer scars. At 14 mo, he remained well, and EGD revealed multiple gastric ulcer scars and biopsy revealed gastritis.

At 26 mo, he remained well, and again EGD revealed multiple gastric ulcer scars and biopsy specimens revealed gastritis (Figure 1B). However, urea breath testing was positive for *H. pylori* infection. Elimination with lansoprazole (30 mg twice daily), amoxicillin (750 mg twice daily) and clarithromycin (200 mg twice daily) for 7 d was successful, and was maintained with rabeprazole (10 mg daily).

At 37 mo, he remained well, but EGD revealed severe active inflammation (edematous mucosa with exudates, multiple ulcers and stricture) at the body of the stomach (Figure 1C). Biopsy revealed no neoplastic infiltrate and RUT was negative. Laboratory results in serum included lactate dehydrogenase 122 IU/L (reference range: 124-226 IU/L), soluble interleukin 2 receptor 325 U/mL (reference range: 190-650 U/mL), carcinoembryonic antigen 3.3 ng/mL (normal value: ≤ 5.0) and amylase 181 IU/L (reference range: 58-167 IU/L). Serum Epstein-Barr antiviral-capsid-antigen IgG titer was 160. Cytomegalovirus (CMV) antigenemia assay and the *Treponema pallidum* hemagglutination test were negative. Additional investigation on reference to other facilities precluded the presence of malignant tumor and inflammatory bowel disease, whereas ultrasonography of the abdomen showed slight enlargement of the pancreatic head. Serum IgG4 at this time was 165 mg/dL (reference range: 4.8-105 mg/dL). The presence of autoimmune pancreatitis (AIP) was suspected.

At 49 mo, he remained well on maintenance treatment with rabeprazole. EGD revealed multiple ulcers and ulcer scars, and worsening of the stricture of the upper stomach (Figure 1D). At 54 mo, in February 2009, he remained well and completely asymptomatic. Repeated EDG disclosed multiple ulcer scars with stenosis of the upper body and well-defined ulcers at the lesser curvature (Figure 2). Pathological examination of gastric lesion biopsies revealed intense infiltration of plasma cells that contained IgG4 (Figure 3). Serum IgG was 1909 mg/dL (reference range: 870-1700 mg/dL); IgG4 was 203 mg/dL (reference range: 4-108 mg/dL); complement component C3 was 114 mg/dL (reference range: 80-140 mg/dL); complement component C4 was 25.9 mg/dL (reference range: 11.0-34.0 mg/dL); and amylase was 167 IU/L (reference range: 58-167 IU/L). Antinuclear antibody, rheumatoid factor, anti SS-A antibody, and anti SS-B antibody were negative. Computed tomography scanning of the abdomen showed that the pancreas was normal, with no evidence of enlargement. Magnetic resonance imaging of the abdomen with magnetic resonance cholangiopancreatography revealed normal biliary and pancreatic ducts, and confirmed the lack of pancreatic enlargement.

Although the high serum IgG4 level strongly suggested AIP, the clinical imaging studies showed no pancreatic enlargement. Corticosteroid therapy and the management of side effects were discussed, but the patient, who was asymptomatic, declined this option. At 64 mo (December 2009), he remains well and stable under maintenance therapy with a proton-pump inhibitor.

DISCUSSION

AIP is a specific type of pancreatitis that is thought to have an autoimmune etiology, and typically shows infiltration of IgG4-positive plasma cells and increased levels of serum IgG4[2,3]. IgG4-positive plasma cells may involve not only the pancreas, but also other organs including the bile duct, gallbladder, salivary gland, thyroid gland, lungs, stomach, colon, liver, retroperitoneum, kidney, prostate and lymph nodes. In 2003, Kamisawa *et al*[4,5] proposed a new clinicopathological entity, IgG4-related sclerosing disease, and suggested that AIP is a pancreatic lesion that reflects this systemic disease. Shinji *et al*[6] described a relationship between gastric ulcer and AIP in 2004, and detected gastric ulcer in eight of 23 AIP patients, and significantly more abundant IgG4-positive plasma cell infiltration in the gastric lesions of AIP patients. They have concluded that AIP is closely associated with gastric ulcer with abundant IgG4-bearing plasma cell infiltration.

A common cause of gastric ulcer is *H. pylori* infection. However, the gastric lesions in the present patient worsened after successful eradication of *H. pylori* with triple therapy. He had no history of non-steroidal anti-inflammatory drug use, which is another important cause of gastric ulcer. Pathological and serological findings precluded the possibility of gastric cancer of the scirrhous type, malignant lymphoma arising from mucosa-associated lymphoid tissue, Crohn's disease, reactivation of CMV infection, chronic active EBV infection, and syphilis. Given his elevated serum levels of IgG4 and abundant infiltration of IgG4-positive plasma cells, we consider that the gastric lesions in this patient were associated with infiltration of IgG4-positive plasma cells, and that the most likely diagnosis is IgG4-related sclerosing disease. Since some IgG4-related sclerosing disease patients relapse and improve spontaneously, worsening of this gastric lesion after *H. pylori* eradication was assumed to be independent of *H. pylori* infection. From recent reports that suggest that IgG4-related sclerosing disease is associated with cancer, our treatment goals in this asymptomatic 77-year-old man are the prevention of gastric cancer and gastric obstruction[7,8]. Since corti-

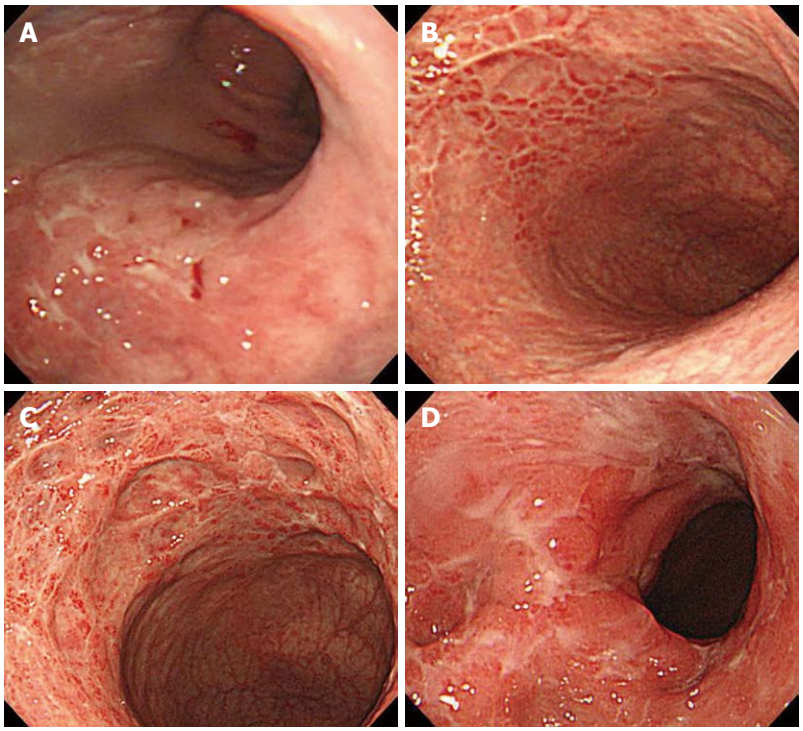


Figure 1 Endoscopic findings show chronic persistent inflammation of the stomach. A: Multiple active ulcers and edematous mucosa in June 2004; B: Multiple scarring ulcers and erythematous mucosa in August 2006; C: Multiple healing and scarring ulcers in July 2007; D: Multiple active ulcers and edematous mucosa with luminal stenosis in July 2008.

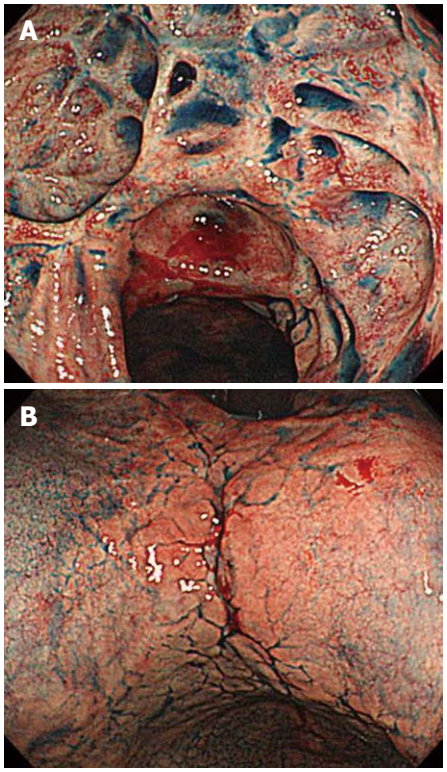


Figure 2 Endoscopic view after indigo carmine dye spray showed luminal deformity and active ulcers in February 2009. A: Multiple scarring ulcers appearing like pseudo-diverticulum in the upper body of the stomach and stenosis of the upper body due to annular scarring ulcers; B: Linear ulcers on the lesser curve of the middle body of the stomach.

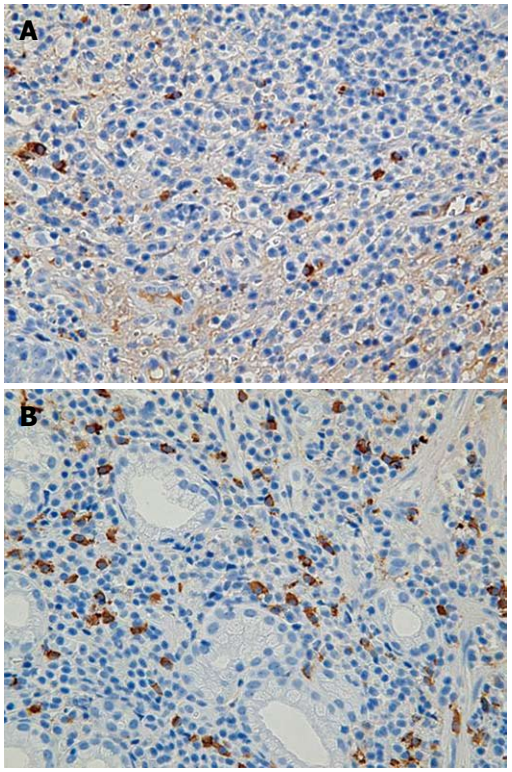


Figure 3 Histopathological findings of biopsy specimens of the stomach. A: Mild infiltration of IgG4-positive plasma cells at the scarring ulcer (15/high power field); B: Severe infiltration of IgG4-positive plasma cells at the active ulcer (50/high power field).

costeroid therapy is effective in IgG4-related sclerosing disease, it may be considered the first treatment option, albeit with consideration to its many side effects, particularly in elderly patients, and uncertainty over optimal dosages. This patient, who was asymptomatic, declined this option and was placed instead on maintenance therapy with a proton-pump inhibitor. Treatment of this

rare gastric ulcer will be advanced by multicenter trials in patients with gastric ulcer and IgG4-related sclerosing disease.

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CASE REPORT

Late retroperitoneal recurrence of hepatocellular carcinoma 12 years after initial diagnosis

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Abstract

Hepatocellular carcinoma (HCC) is an aggressive tumor with poor long-term prognosis. Here, we present an unusual patient with a solitary recurrence of HCC in the right kidney 12 years after the initial diagnosis. This illustrates the importance of considering late recurrence in patients with a history of HCC and the management of these metastases.

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Key words: Hepatocellular carcinoma; Late recurrence; Metastasis; Retroperitoneal

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Wong TCL, To KF, Hou SSM, Yip SKH, Ng CF. Late retroperitoneal recurrence of hepatocellular carcinoma 12 years after initial diagnosis. *World J Gastroenterol* 2010; 16(17): 2187-2189 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i17/2187.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i17.2187

INTRODUCTION

Hepatocellular carcinoma (HCC) is an aggressive tumor with poor long-term prognosis. The reported 5-year recurrence rate ranges from 75% to 100%[1]. Therefore, extrahepatic metastasis of HCC is uncommon due to the highly malignant nature of the primary tumor. However, with advances in different treatment modalities for HCC, the incidence of extrahepatic metastasis appears to be increasing[2]. Nevertheless, most recurrences occur relatively early after the initial diagnosis and treatment. Here, we report a patient who presented with a renal tumor 12 years after the initial treatment of primary HCC, which turned out to be a solitary recurrence of the initial HCC. The management of these distant metastases is also highlighted.

CASE REPORT

A 61-year-old man who was a chronic hepatitis B carrier with Child's A liver cirrhosis, first presented 12 years ago with hemoperitoneum secondary to ruptured HCC. Emergency laparotomy for hemostasis was performed. Non-anatomical resection of the liver tumor was subsequently performed 1 mo later after his condition was optimized. Pathology showed an 8 cm × 6 cm × 4 cm, moderately differentiated HCC with a clear resection



Figure 1 Computed tomography showing the contrast-enhanced mass in the right kidney.

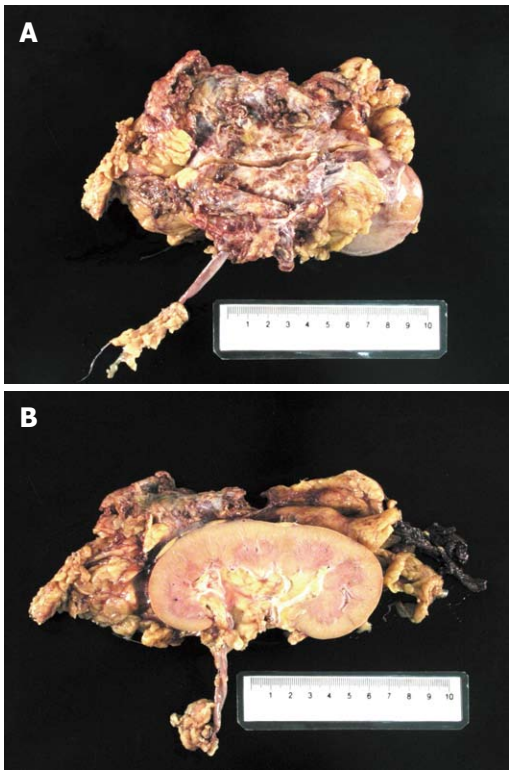


Figure 2 Gross specimen of tumor showing its relationship with the right kidney. A: External appearance; B: From cut section of kidney.

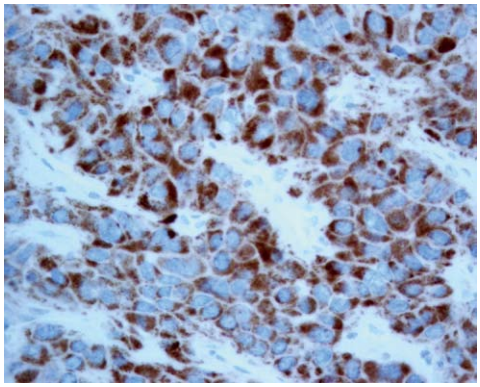


Figure 3 Immunological stain of the tumor with HepPar, confirmed the tumor as hepatocellular carcinoma.

margin. The postoperative serum α fetoprotein (AFP) level dropped from 1500 to 2 ng/mL.

He was followed up regularly, 3-monthly for the first 2 years, followed by half-yearly follow-up. Serum AFP was measured during each follow-up and abdominal ultrasound was also performed during each follow-up for the first 5 years and then yearly during subsequent follow-up. There was no evidence of recurrence and the patient remained well clinically until 12 years later, when surveillance ultrasound revealed a right renal mass. Subsequent computed tomography showed a 5.3 cm pedunculated, well-circumscribed, contrast-enhanced soft tissue tumor in the right kidney, suspicious of renal cell carcinoma (RCC) (Figure 1). There was no space-occupying lesion in the liver or ascites. The tumor was asymptomatic and was not found on the last ultrasound performed 1 year ago. Serum AFP level was also increased from 3 (6 mo previously) to 27 ng/mL. However, in view of the long recurrence-free period and no evidence of local recurrence, the treatment plan was to closely monitor the raised AFP level and proceed to treatment of the renal mass.

With the past history of laparotomy and hepatectomy, retroperitoscopic nephrectomy was planned. During the operation, a large vascular, friable and exophytic tumor was found to have developed in the right kidney. However, the tumor was quite adhesive to the surrounding tissue, such that it inadvertently ruptured despite careful dissection. The surgery was then converted to an open procedure. The operation lasted 195 min with an estimated blood loss of 3 L. The post-operative course was uneventful and he was discharged 7 d after the operation.

However, the subsequent unexpected pathology was found to be extra-hepatic metastasis of HCC at Gerota's fascia. Macroscopically, the tumor was in the perinephric fat with no invasion to the renal capsule (Figure 2). Microscopic examination showed trabeculae of polygonal eosinophilic tumor cells, with epithelial cohesion, marked pleomorphism, associated with sinusoidal-type vascular channels and bile canaliculi were also identified. The kidney and pelvi-calyceal system were intact. When compared with the previously resected HCC 12 years ago, they both demonstrated similar microscopic characteristics and both were immunoreactive to polyclonal carcinoembryonic antigen and HepPar (Figure 3), but not to markers for RCC. Both the primary and recurrent HCC demonstrated the same TP53 mutation in exon 7 (R249S AGG > AGC). This result by itself was not confirmatory, but supported the clonal relationship of the primary and recurrent HCC in this case.

After nephrectomy, the patient's serum AFP level dropped to 1 ng/mL, i.e. normal. As there was no evidence of other systemic recurrence, no adjuvant chemotherapy was administered. The patient remained well 18 mo after the nephrectomy and serum AFP remained normal. The latest positron-emission tomography per-

formed at 1 year after nephrectomy showed no evidence of recurrence.

DISCUSSION

In the past, extrahepatic metastasis of HCC was considered a relatively uncommon phenomenon, owing to the highly malignant nature of the primary tumor. Nonetheless, with advances in treatment for HCC, its overall prognosis is improving and the incidence of extrahepatic metastasis appears to be increasing[3]. The commonest sites of extrahepatic metastases are lung, lymph node and bone followed by adrenal glands[4-6]. Extrahepatic metastasis can occur *via* three routes: hematogenous (56%), lymphogenous (27%) and direct invasion (22%) and more than one mode of spread can occur[7]. It is uncommon for HCC to spread to kidney and most believe that tumor spread to the kidneys is *via* the hematogenous route[8]. In our patient, the metastasis spread to the perinephric tissue without direct invasion of kidney parenchyma. It was postulated that the initial rupture of the primary liver tumor resulted in some seeding of tumor cells in the retroperitoneal region giving rise to subsequent tumor recurrence.

However, it is still extremely rare to have recurrent HCC 12 years after the initial diagnosis and treatment. The reported median duration between initial treatment for HCC to extrahepatic spread is 23.2 mo[1]. While there was a report suggesting that HCC can recur more than 6 years after initial management[9], there are no reports of recurrence occurring 10 years after initial treatment. It is difficult to explain the exceptionally long time lapse between treatment of the primary tumor and the current recurrence in our patient. It can not be explained even by shed cells belonging to a very low malignant clone of tumor cells.

Although there is no standard management protocol for patients with extrahepatic metastasis of HCC, it is generally believed that aggressive treatment for extrahepatic metastasis can prolong survival in selected patients with good performance status and limited volume of recurrence. In a review of patients with both intrahepatic and extrahepatic recurrent HCC, patients who received an aggressive combination of metastatectomy and locoregional therapy, with or without systemic therapy, had a better survival rate compared to those who received only non-surgical treatment (median survival after recurrence was 44.0 mo in the aggressive treatment group *vs* 10.6 mo in the non-surgically treated group)[2]. For patients with lung metastasis, it was also shown that a longer disease-free interval from primary treatment and a smaller number of metastasis (less than three) were associated with better prognosis[10]. In our patient, the solitary nature and long disease-free interval were relatively good prognostic factors.

Certainly, a more frequent follow-up protocol, especially imaging, may help to detect the lesion at an earlier stage. However, a careful balance between the yield and medical cost is needed, as late recurrence of HCC after a disease-free period of 5 years is still a very rare condition.

Our case illustrates that, in managing patients with a past history of HCC who present with new mass lesions, the diagnosis of extra-hepatic metastasis should always be suspected. Although the final treatment might not be altered, the diagnosis of metastatic HCC would allow better surgical planning and counseling for the patient.

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CASE REPORT

Partial intestinal obstruction secondary to multiple lipomas within jejunal duplication cyst: A case report

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Abstract

Lipoma within jejunal duplication presenting as abdominal bloating and partial intestinal obstruction is an exceptional clinical entity. We report a case of 68-year-old man complaining of abdominal bloating for 10 d due to multiple lipomas arising from jejunal duplication cysts. Only a few cases of a single lipoma within a Meckel's diverticulum giving rise to this clinical scenario have been reported in the English language literature. However, no case of multiple lipomas within jejunal duplication cysts has been reported. We present a case in which double-balloon endoscopy revealed a small intestinal structure changed into Meckel's diverticulum-like cavities containing several lipomas. This case highlights intestinal lipoma as an uncommon cause of adult intussusceptions, which should be included in the differential diagnosis of small intestinal obstruction and appropriate examinations should be chosen.

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Key words: Duplication cyst; Lipoma; Double-balloon endoscopy; Jejunum; Intestinal obstruction

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INTRODUCTION

Lipoma of the small intestine is a benign tumor of mesenchymal origin which is mostly found by chance during gastrointestinal investigation. Invaginations account for 2/3 of small bowel occlusion caused by up to 80% of tumors and the lipoma is the most frequent benign tumor that causes invagination in its submucous polypoid and it is in more or less scissile form[1].

However, multiple lipomas within the intestinal duplication canal as a predominant cause of partial intestinal obstruction is an exceptional clinical scenario. We report a case of a 68-year-old man with a 10-d history of abdominal bloating after meals caused by intussusception due to a small intestine lipomatous lesion located in the jejunal duplication cyst. Although the same complications such as intestinal obstruction and intussusception may crop up in the duplication patients, we still consider the multiple lipomas as the main cause of the symptom.

CASE REPORT

A 68-year-old man was referred to our department complaining of gradual abdominal bloating after meals for the last 10 d, but without abdominal pain and vomiting. He had a bowel movement every 2 d despite a history of chronic constipation for 6 years. The patient refused eating for 2 d and lost approximately 5 kg. There was no

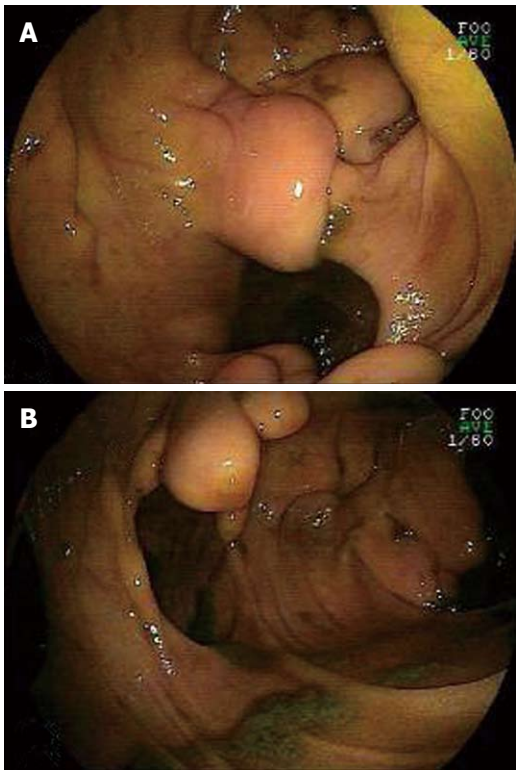


Figure 1 Double-balloon endoscopy (DBE) by the oral approach revealing several rounded protuberances in the jejunum (B) and a diverticulum-like hole (A).

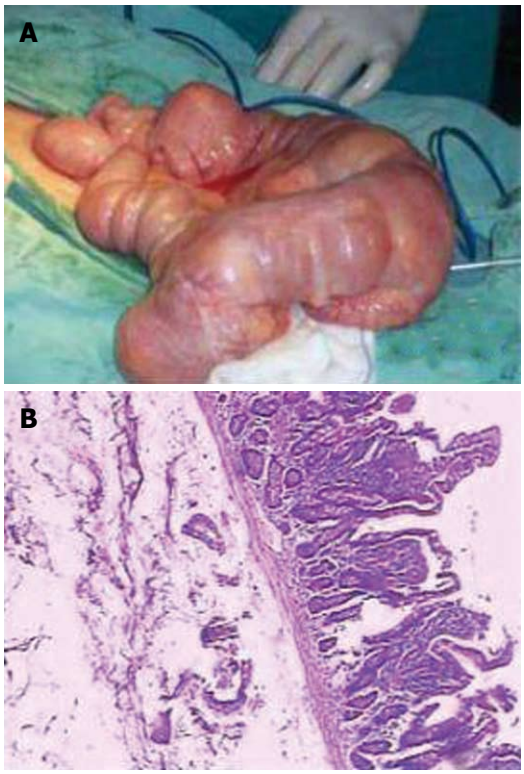


Figure 2 Resected specimen of jejunal tissue. A: Macroscopic appearance of the resected specimen with seven remarkably dilated cysts; B: Microscopic appearance of resected tissue showing multiple lipomas lining within intestinal wall (HE, × 20).

more relevant past medical, family or surgical histories. Physical examination revealed abdominal bulge with visible intestinal peristalsis, and active bowel sounds. Abdominal palpation revealed a firm, round and fixed mass of 7 cm × 4 cm over the left umbilical region. Examination of other systems was unremarkable with insignificant laboratory results.

Abdominal plain X-ray film showed air-fluid levels while colonoscopy failed to reveal any lesions which might be responsible for the symptoms. However, double-balloon endoscopy (DBE) revealed small intestinal structures which changed into Meckel's diverticulum-like cavities at 30 cm beyond the ligament of Treitz from the oral route (Figure 1A). Several yellow spherical or stalactite-like protuberances of unequal size rose into the jejunal cavity in these cystic formations with smooth surface (Figure 1B). We also found scar tissues in the partial jejunal cavity (Figure 1A). A possible diagnosis of jejunal duplication with multiple lipomas was established and the patient underwent exploratory laparotomy. One remarkably dilated intestinal canal with seven duplication cysts of about 7.5 cm × 6 cm on the mesenteric aspect of the jejunum of around 55 cm was found intraoperatively (Figure 2A). All the cyst-like dilatation had independent vascular supply, some of them were connected to the intestinal cavity while some did not. The end of intestinal tract invaginating into the low intestinal canal appeared dark red and edematous, while the upper segment was dilated significantly. Excision of the cystic lesion and an end-to-end anastomosis of the jejunum were performed. Pathological examination of the resected specimen revealed jejunal duplications having seven cysts ranging from 5.5 cm × 6 cm to 8.5 cm × 7.5 cm, with multiple submucosal lipomas lining on its wall (Figure 2B). The biggest lipoma was 32 mm in diameter. The patient had an uneventful postoperative recovery and was discharged on the seventh post-operative day. He remained well 14 mo after surgery without any symptoms of recurrence.

DISCUSSION

Alimentary tract duplications are rare congenital malformations, which in most of cases are diagnosed in infancy and childhood, although they can remain undetected until older age[2]. In contrast to Meckel's diverticulum, duplication cysts are located on the mesenteric aspect and share a common smooth muscle wall and vascular supply[2,3]. Most of the patients are asymptomatic and remain undiagnosed for years. When symptomatic, they manifest as bleeding, constipation and obstruction as a result of direct compression, volvulus or intussusception[4,5]. Our patient had a history of chronic constipation as a presenting symptom of jejunal duplication. Although it seemed to be the complication of duplication cyst, the right diagnosis was only made after 6 years.

Lipoma is a benign tumor of mesenchymal origin which is uncommon to be localized in the small bowel.

It is mostly encountered incidentally during the investigation of the gastrointestinal tract for other reasons, since they are usually asymptomatic[6]. Generally, lipoma is defined to originate in the submucosal layer and usually solitary (85%-90%), with variable sizes ranging from 1 to 30 cm[7]. Zografos *et al*[8] reported that lesions smaller than 1 cm are generally unable to cause symptoms, while 75% of those > 4 cm might give rise to gastrointestinal symptoms. Intestinal occlusion is one of the major results, which is caused by direct pressure by the lipoma or due to intestinal intussusception. Duplication cysts and lipomas can both lead to intussusception or obstruction, but in this case, we speculated that the small bowel lipomas should be the main reason. Firstly, more than 60% of the gastrointestinal duplications are diagnosed prenatally or in the first 2 years of life[9], making it rare to be symptomatic in adults. Ileocecal valve makes cystic duplications easier to cause obstruction of the colon than jejunal duplication. Secondly, several lipomas in the lesion are greater than 3 cm in diameter which might be the cause of intussusception and partial intestinal obstruction in our patient. Direct compression of cystic duplication led to intestinal stenosis, prolonging the time for chyme passage. Consequently, jejunal lipomas continued to be under progressive inflammatory stimulation.

Preoperative diagnosis of duplication cyst or small intestinal lipoma is difficult. Data from our hospital shows that, from 1998 to 2008, only 14.3% of duplication cysts have been correctly diagnosed before surgery. Abdominal computed tomography and ultrasonography were helpful in identifying large lesions but not in small intestinal lesions. However, the availability of DBE[10] and wireless capsule endoscopy made it possible to directly examine the whole digestive tract. In this case, we found multiple small intestinal lipomas arising from the spherical type duplication cysts of the jejunum using DBE. From April 2007 to May 2009, 214 enteroscopic studies were carried out, and 165 patients underwent entire digestive tract examinations. We found other two cases of tubular duplications of the ileum.

Although it is a rare entity, malignant transformation has been reported, mostly to adenocarcinoma[11]. From our patient, we have found out that jejunal duplication may induce the growth of lipoma, hence causing symptoms of intestinal obstruction. We consider that surgical removal of the lesion in asymptomatic cases is necessary because of the risk of malignant degeneration.

Multiple lipomas within jejunal duplication cysts are rare and difficult to diagnose before surgery. This case highlights intestinal lipoma as an uncommon cause of adult intussusceptions, which should be included in the differential diagnosis of small intestinal obstruction. Such a case found by DBE is an example to remind doctors to be vigilant in diagnosing patients with intestinal obstruction and in choosing appropriate examinations.

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BOOK REVIEW

Rectal prolapse: Diagnosis and clinical management

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Abstract

The exact cause of rectal prolapse is not well addressed, but it is often associated with long standing constipation, advanced age, chronic obstructive pulmonary disease and some neurological disorders. Rectal prolapse is usually only a symptom, which needs a focus on discovery of the underlying pathology or disorder. Three different clinical presentations are often combined and called rectal prolapse. Rectal prolapse can be divided into full thickness rectal prolapse where the entire rectum protrudes beyond the anus, mucosal prolapse where only the rectal mucosa (not the entire wall) prolapses, and internal intussuception wherein the rectum collapses but does not exit the anus. Although constipation and straining may contribute to the development of rectal prolapse, simply correcting these problems may not improve the prolapse once it has developed. There are many different approaches to surgical correction of rectal prolapse.

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Key words: Rectal Prolapse; Procidentia; Complete prolapse

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INTRODUCTION

Rectal prolapse, or procidentia or "complete prolapse", defined as a protrusion of the rectum beyond the anus[1], occurs at the extremes of age. In the pediatric population, the condition is usually diagnosed by the age of 3 years, with an equal sex distribution. In the adult population, the peak incidence is after the fifth decade and women are more commonly affected, representing 80%-90% of patients with rectal prolapse[2].

Complete rectal prolapse is a disabling condition that has been reported even since the Egyptian and Greek civilizations[3]. Throughout the history of medicine, multiple approaches to the treatment of rectal prolapse have been described. In the past century, its management has evolved a great deal due to accumulation of knowledge obtained from physiologic investigations and follow up of surgical series[4].

The surgical landscape for rectal prolapse has expanded to include new treatment modalities such as the STARR and EXPRESS procedures. However, technical details, indications, and outcomes of these new techniques are not widely understood. The richness and variety of choices for treating rectal prolapse may become confusing, and controversial. These are some reasons why many surgeons feel the need for one articulate and comprehensive volume that present an all inclusive understanding of the pathophysiology of rectal prolapse and state of the art surgical treatment for it.

BOOK REVIEW

The book "Rectal Prolapse: Diagnosis and Clinical Management, edited by Altmomare and Filippo Pucciani (PP 206), illustrated, Springer Publisher (2008), ISBN:

978-88-470-0688-6" offers a body of information encompassing all aspects of rectal prolapse. It deals comprehensively with the various forms of prolapse, including external prolapse, rectal intussusception and genital prolapse. The chapters on etiology and investigation set out in detail the present position regarding the value of the advancements in clinical practice. Beside the classic operations, new treatment modalities such as the STARR and EXPRESS procedures are dealt with, and their indications are considered in relation to the clinical presentation and the various other options. Function following surgery receives considerable attention and the difficult problems that may be posed by recurrence after surgery are dealt with. Non surgical treatment and rehabilitation are also described in this book.

The book, is beautifully laid out with very clear illustrations including high quality operative color photographs and line drawings. It is important not only to surgeons but also to gastroenterologists, physiologists and radiologists.

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Meetings

Events Calendar 2010

January 25-26
Tamilnadu, India
International Conference on Medical Negligence and Litigation in Medical Practice

January 25-29
Waikoloa, HI, United States
Selected Topics in Internal Medicine

January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology Conference

January 28-30
Hong Kong, China
The 1st International Congress on Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of Gastroenterology & Endoscopy Meeting

March 09-12
Brussels, Belgium
30th International Symposium on Intensive Care and Emergency Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian National Association for Study of the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on Diabetes PACD14

March 25-28
Beijing, China
The 20th Conference of the Asian Pacific Association for the Study of the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology and Hepatology Conference, EGHC 2010

April 14-17
Landover, Maryland, United States
12th World Congress of Endoscopic Surgery

April 14-18
Vienna, Austria
The International Liver Congress™ 2010

April 28-May 01
Dubrovnik, Croatia
3rd Central European Congress of surgery and the 5th Croatian Congress of Surgery

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual Meeting

May 06-08
Munich, Germany
The Power of Programming: International Conference on Developmental Origins of Health and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and Rectal Surgeons Annual Meeting

June 04-06
Chicago, IL, United States
American Society of Clinical Oncologists Annual Meeting

June 09-12
Singapore, Singapore
13th International Conference on Emergency Medicine

June 14
Kosice, Slovakia
Gastro-intestinal Models in the Research of Probiotics and Prebiotics-Scientific Symposium

June 16-19
Hong Kong, China
ILTS: International Liver Transplantation Society ILTS Annual International Congress

June 20-23
Mannheim, Germany
16th World Congress for Bronchoesophagology-WCBE

June 25-29
Orlando, FL, United States
70th ADA Diabetes Scientific Sessions

August 28-31
Boston, Massachusetts, United States
10th OESO World Congress on Diseases of the Oesophagus 2010

September 10-12
Montreal, Canada
International Liver Association's Fourth Annual Conference

September 11-12
La Jolla, CA, United States
New Advances in Inflammatory Bowel Disease

September 12-15
Boston, MA, United States
ICAAC: Interscience Conference on Antimicrobial Agents and Chemotherapy Annual Meeting

September 16-18
Prague, Czech Republic
Prague Hepatology Meeting 2010

September 23-26
Prague, Czech Republic
The 1st World Congress on Controversies in Gastroenterology & Liver Diseases

October 07-09
Belgrade, Serbia
The 7th Biannual International Symposium of Society of Coloproctology

October 15-20
San Antonio, TX, United States
ACG 2010: American College of Gastroenterology Annual Scienitfic Meeting

October 23-27
Barcelona, Spain
18th United European Gastroenterology Week

October 29-November 02
Boston, Massachusetts, United States
The Liver Meeting® 2010--AASLD's 61st Annual Meeting

November 13-14
San Francisco, CA, United States
Case-Based Approach to the Management of Inflammatory Bowel Disease

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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

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/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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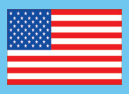
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EDITORIAL

Serum response factor: Look into the gut

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Abstract

Serum response factor (SRF) is a transcription factor that regulates many genes involved in cellular activities such as proliferation, migration, differentiation, angiogenesis, and apoptosis. Although it has only been known for about two decades, SRF has been studied extensively. To date, over a thousand SRF studies have been published, but it still remains a hot topic. Due to its critical role in mesoderm-derived tissues, most of the SRF studies focused on muscle structure/function, cardiovascular development/maintenance, and smooth muscle generation/repair. Recently, SRF has received more attention in the digestive field and several important discoveries have been made. This review will summarize what we have learned about SRF in the gastrointestinal tract and provide insights into possible future directions in this area.

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Key words: Angiogenesis; Cell invasion; Myofibroblast differentiation; Smooth muscle contraction; Serum response factor; Wound healing

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INTRODUCTION

Although serum response factor (SRF) has only 25-year history, its studies have been exponentially grown in several fields including smooth muscle structures, cardiac functions, cellular stress responses and cell motility. SRF is a ubiquitously expressed transcription factor, therefore, its role should be far beyond these areas. When the new millennial dawn broke, we opened a new field for SRF research-digestive system. Several important discoveries have been made in different parts of the system ever since, which foresee a bright and fruitful future for this area. This article is to provide you an update in this line of study and hopefully point you to the right direction.

HISTORY OF SRF

SRF was first identified by Treisman[1] in 1986 based on a previous observation in Greenberg's lab that resting cells responded to serum addition with a rapid activation of *c-fos*[2]. He discovered that it is SRF that initiates the immediate response of c-fos to serum or any other growth factors by binding to a short DNA sequence-serum response element (SRE), which is located about 300 bp upstream of the *c-fos* gene transcription initiation site. Since then, SRE has been identified in as many as 300 human genes, accounting for 1% of our entire genome[3,4]. Although it has only been known for a little over two decades, studies on SRF have been populated exponentially. Last year, more than a hundred SRF studies were documented in PubMed; and ten papers have already been published within the first 3 wk of this

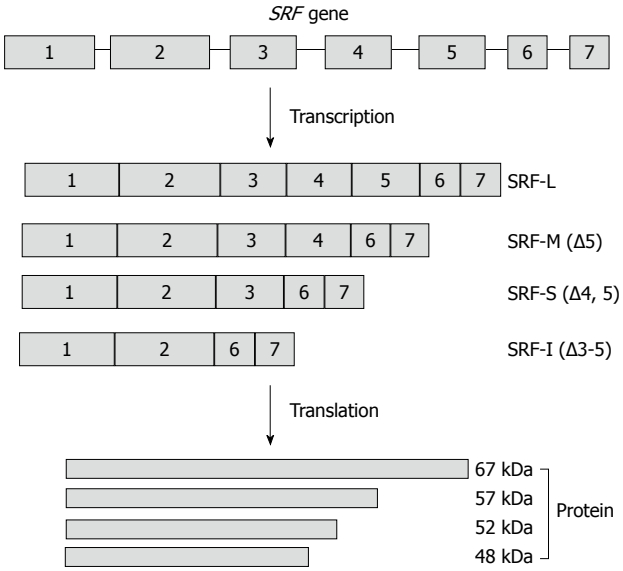


Figure 1 Serum response factor (SRF) splice variants (adapted from Chai and Tarnawski 2002).

year. Most of the published SRF studies deal with its functions in muscle structures or in the regulation of immediate early genes. However, SRF is a ubiquitously expressed protein and thus its role must be far beyond these areas. A few years ago, we started to look for SRF in the gastrointestinal (GI) tract[5,6]. After that, several other groups have expanded our mission. Today, there have been over 20 studies published dealing with SRF in the digestive system.

BIOCHEMISTRY OF SRF

The human *SRF* gene was mapped to chromosome 6p21.1. It is 10607 bp long and contains 7 exons. In both humans and mice, SRF can be expressed in different isoforms due to alternative splicing and some of them appear to display tissue specificity[7]. For instance, SRF-S, which lacks both exon 4 and 5 (Δ4, 5), has only been detected in the aorta, while SRF-I, which is the shortest isoform (missing exon 3, 4 and 5), is specific to embryonic tissues. On the other hand, SRF-M, which lacks only exon 5, has been shown to be a dominant negative mutant (Figure 1).

Full length SRF protein, which is approximately 67 kDa, was shown to contain three distinct domains: a SRE DNA binding domain, a transactivation domain and multiple phosphorylation sites[8]. The DNA binding domain, which also serves for dimerization and interaction with accessory factors, has been highly conserved throughout evolution, showing a 93% homology between fruit flies and humans[9]. Phosphorylation at Serine 103, which is immediately adjacent to the DNA binding domain, was shown to greatly enhance SRF activity[10]. Since its initial discovery in response to serum, SRF has also been shown to be activated by several other agents, including mitogens, cytokines, specific oncogenes and extracellular stimuli, such as antioxidants, UV light and microgravity, to name a few[7].

FUNCTIONS OF SRF

SRF is a master regulator of many cellular activities including cell growth and differentiation, cell migration, and apoptosis. To date, approximately 300 human genes have been estimated to contain an SRE element and be activated by SRF, accounting for 1% of our entire genome[3,4]. Early transgenic data provided important clues to some of the biological functions of SRF, best elucidated through its role in the myocardium, which is of mesodermal origin, and to the different optimal expression requirements during embryogenesis and adulthood[7]. More specifically, mice with complete SRF knockout (*srf* -/-) failed to develop the mesoderm and died in the uterus between E8.5 and E12.5[11], indicating that SRF is required for early embryonic development. For this reason, we generated a mouse model with overexpression of a dominant mutant SRF in cardiac-specific tissue and found that SRF is required for myofiber generation as the transgenic mice died within the first week after birth due to heart dysfunction[12]. For comparison, we also developed a mouse model with overexpression of functional SRF in the heart and demonstrated that too much SRF can cause hypertrophic cardiomyopathy as the mice died of heart failure within 6 mo[13]. From these initial studies, SRF emerged as a key factor in muscle development and maintenance. In addition, modulation of SRF expression levels seems to play an important role in its different functions, where high expression levels of SRF are required for proper embryonic development, while lower levels may be more beneficial in adulthood[7].

IMPLICATIONS IN GI

Even though SRF had been studied extensively in other tissues since its discovery, its role in the GI system was not examined for at least another decade. The earliest record that can be found was in 1997, and this study showed that SRF binding activity is elevated in the liver of Long-Evans Cinnamon rats (animal model of Wilson's disease) compared to Wistar rats[14]. In addition, several other studies used GI-derived cell lines purely as tools to investigate the molecular properties of SRF[15-17]. However, the role of SRF in the GI system was not studied directly until eight years ago, when our group found that SRF is not only expressed in smooth muscle structures, such as muscularis mucosa and muscularis propria, which are of mesoderm origin, but it is also found at intermediate expression levels in the mucosal epithelium, which is of endoderm origin[5]. Since then, work from our group and others has provided important information about the role of SRF in both normal and pathological processes in the digestive system (Table 1).

Esophagus

Esophageal ulcers occur with a great geographical variation, from 5%-10% in the United States to approximately 80% in some Iranian regions. Its causes are also different with locations. While gastroesophageal reflux is its main cause in

Table 1 Identified roles of SRF in the GI tract

GI system	Process involved	Molecules associated	GI disorder associated
Esophagus	Myofibroblast differentiation[19]	TGFβ, ILK	Ulcer
Stomach	Angiogenesis[20]	VEGF, Rho-actin, MEK-ERK	Ulcer
Stomach	Re-epithelialization, muscular structure restoration[6]	Actin, immediate-early genes	Ulcer
Stomach	*H. pylori* activates SRF[21,22]	CagA, villin	Intestinal metaplasia
Intestine	Smooth muscle contraction[23,24]	Smooth muscle actin, smooth muscle myosin, smoothelin, F/G actin	Intestinal obstruction, CIPO
Colon	Alternative splicing, cell survival[26]	SRFΔ5, K-ras	Colon cancer
Liver	Cell cycle; hepatocyte proliferation/survival[29,30]	IGF-1	Liver injury
Liver	Cell proliferation, cell cycle, apoptosis[33]	E2F1	Hepatocellular carcinoma
Liver	Cell invasion[31]	E-cadherin, β-catenin	Liver metastasis
Pancreas	Cell proliferation[32]	Pro-inflammatory cytokines	Pancreatitis

SRF: Serum response factor; GI: Gastrointestinal; TGF: Transforming growth factor; VEGF: Vascular endothelial growth factor; MEK: Mitogen-activated protein kinase kinase; ERK: Extracellular regulated kinase; *H. pylori*: *Helicobacter pylori*; IGF: Insulin-like growth factors; CIPO: Chronic intestinal pseudo-obstruction.

the United States, in Europe it is alcohol consumption and in the Middle East it is the diet[18]. Healing of esophageal ulcers proceeds *via* a series of overlapping events[19], and among them myofibroblasts make a significant contribution to the wound closure. Our study[20] showed that when the connective tissue has been damaged and denuded of its epithelium during gastrointestinal ulceration, fibroblasts next to the ulcer area are activated to become myofibroblasts and participate in restoration of new epithelial continuity and extracellular matrix. Over-expression of SRF promotes myofibroblast differentiation both *in vitro* and *in vivo*, and knockdown of SRF was sufficient to prevent TGFβ-induced myofibroblast differentiation.

Stomach

While there are nearly 50 thousand publications dealing with stomach ulcers, we are the only researchers to have investigated the role of SRF in this common gastric disorder. SRF is a master regulator of cytoskeleton dynamics and cell motility. We showed that injury-activated SRF is critical to gastric ulcer healing, as local knockdown of SRF severely impairs angiogenesis[21], an essential process for any wound healing. Without SRF, VEGF, the most powerful activator of angiogenesis, loses its power. Since angiogenesis is a key step in tumor progression by providing growing tumors with oxygen and nutrient supplies through generation of new blood vessels, these findings may have potentially important therapeutic implications for blocking cancer progression. We also demonstrated[6] that over-expression of SRF in gastric epithelial cells or in smooth muscle cells (*in vitro*) as well as in gastric tissue (*in vivo*) can promote cell proliferation/migration, and thereby promotes re-epithelialization and restoration of smooth muscle structures damaged by ulcers. These findings show great potential for therapeutic applications of SRF.

While the normal processes of angiogenesis and wound healing have been indirectly associated with promoting cancer when inappropriately activated, therefore making SRF a potential oncogenic factor through its regulation of these processes and a promising target for cancer therapy as elucidated before, more direct evidence that SRF can indeed promote cancer progression has come from different sources. First, two different groups linked SRF to *Helicobacter pylori* (*H. pylori*), a gram-negative bacterium that colonizes the human gastric mucosa, resulting in stomach disorders such as chronic gastritis, peptic ulcers and gastric adenocarcinoma. Of the two *H. pylori* strains, the type one strain contains the cag pathogenicity island (PAI), which confers greater virulence compared to the type two strain lacking PAI. Hirata *et al*[22] showed that transfection of the *CagA* gene into gastric epithelial cells greatly increases *in vitro* binding activity of SRF to SRE. Up to that point, CagA protein had only been linked to cellular cytoskeletal rearrangements, after activation through tyrosine phosphorylation. Therefore, aside from linking SRE and SRF to *H. pylori* pathogenesis, their findings are important for understanding *H. pylori* infection mechanisms by identifying a novel, phospho-tyrosine-independent, mode of action of CagA protein. Rieder and co-workers later built on the story by identifying villin as a new target of SRF and showing that SRF mediates Helicobacter-induced intestinal metaplasia in the stomach through villin[23]. Intestinal metaplasia is a premalignant precursor lesion to several organs of the GI tract, including stomach, gall bladder and pancreas, and it is defined by the presence of intestine-like cells expressing enterocyte-specific markers, such as villin.

Intestine

The importance of SRF in the GI tract was further strengthened by the work from Angstenberger and collaborators on smooth muscle contraction[24], which is a key feature of proper GI function. They developed an inducible mouse model where SRF was conditionally knocked out only in the smooth muscle cells of adult mice. The mutant mice developed symptoms of ileus paralyticus due to impaired contraction of intestinal smooth muscle and died 2 wk after the induction. Through more detailed phenotypic and gene expression analysis of the same model system in collaboration with Feil, Mericskay and co-workers confirmed[25] that SRF plays a central role in maintaining proper smooth muscle function and they provide an inducible mouse model that could have potential implications for studying chronic intestinal pseudo-obstruction

(CIPO). The mutant mice displayed cachexia and autopsy showed severe dilation of the intestinal tract associated with food stasis, indicating impairment of GI motility due to smooth muscle deficiency. Defects in GI contractile function can lead to a variety of different disorders, from common and relatively benign, to more rare but potentially life-threatening. CIPO falls in the latter category, sharing similarities with chronic heart failure in the way that the "intestinal pump" is no longer able to effectively maintain tone and coordinate transit of intestinal contents through the luminal cavity, resulting in intestinal pseudo-obstruction[26]. Several different mechanisms have been associated with CIPO, with both genetic and environmental causes, surely making CIPO a multifactorial condition. Nonetheless, these two studies clearly show that SRF plays a central role in proper smooth muscle contraction and that it could be an important model system to shed new light on CIPO. In their editorial commentary, De Giorgio *et al*[26] propose three different models by which SRF ablation could affect intestinal contractility: "(1) loss of smooth muscle cell (SMC) contractile phenotype due to an impairment of the contractile apparatus; (2) degeneration of SMCs with synthetic phenotype; and (3) derangements of pathways (including neuronal ones) implicated in smooth muscle contraction"[26]. Even though results from Angstenberger and Merickskay made it possible to define these models of potential SRF involvement in CIPO, there is definitely room for additional work, especially in regard to the latter model, which is only briefly touched upon by Merickskay and co-workers.

Colon

As we mentioned earlier, SRF can be expressed in different isoforms in a tissue-specific manner due to alternative splicing of mRNA. Patten and co-workers[27] found that the predominant SRF isoform expressed in colon cancer cell lines derived from poorly differentiated tumors (WiDr, HCT116, LoVo, and SW480) is SRFΔ5, the dominant negative isoform lacking the transactivation domain (Figure 1). SRFΔ5 is normally expressed at high levels in terminally differentiated tissues, such as brain, heart, skeletal muscle, testes and liver. However, aberrant elevated expression of SRFΔ5 in other tissues has been associated with medical conditions. For instance, while normal lungs express very low levels of SRFΔ5, hypoplastic lungs, in which stretching is compromised, express elevated levels of this isoform[28]. Similarly, over-expression of another isoform (SRFΔ4,5), which was shown to inhibit transcription of SRF-dependent cardiac muscle genes, was detected in failing hearts[29]. Patten and co-workers also showed that stable expression of SRFΔ5 in rat intestinal epithelial cells (IEC-6) significantly increased cell survival rates, possibly by preventing apoptosis, as cell proliferation was improved only after day 11 and mRNA levels of pro-apoptotic caspase 3 and Fas were significantly reduced.

Liver

It is known that the liver has a remarkable capacity to regenerate after injury. Latasa *et al*[30] found that SRF and its targeted immediate early genes are rapidly activated after partial hepatectomy in rodents. When they knocked down SRF in the liver, this regeneration capacity was severely damaged. Following up on this idea, Sun and co-workers showed that liver-specific SRF knockout in mice led to a lower survival rate, where surviving animals were generally smaller with smaller and poorly functioning livers[31]. Through gene array analysis of SRF deficient liver fragments, they also showed that loss of SRF prevents activation of a wide array of genes, particularly those involved in IGF-1-mediated cell cycle control, consistent with impaired normal growth, as well as several genes specific to hepatocyte function, suggesting that adequate amounts of SRF are indispensable for proper liver development and function. These findings highlight the different expression requirements for SRF in tissue development and proper function/maintenance, stressing the importance of optimal SRF expression. While cell culture and animal models on the mechanistic action of SRF are quite informative, correlation with cancer progression in patients is often determined based on differential expression between normal and tumor tissue, which implies that up- or down-regulation of a particular gene (or aberrant expression of a different variant of the gene) confers more tumorigenic potential to the cell and is therefore maintained. In this respect, Choi *et al*[32] recently reported that nuclear SRF staining, which was not detected in normal colon tissue, was found in 37% of primary colon cancers and 60% of metastatic liver cancer. A similar trend was observed with loss of E-cadherin expression (14% and 33%, respectively), while nuclear expression of β-catenin was significantly higher in primary tumors (56%) compared to normal tissue but did not change much in metastatic tumors. Loss of E-cadherin expression and translocation of β-catenin from the membrane to the nucleus are fundamental steps in disruption of epithelial cell junctions and acquisition of more migratory potential, which are at the basis of tumor metastasis. Therefore, to follow up on these observations, Choi and co-workers showed that over-expression of SRF in colorectal carcinoma cells enhanced cell motility and invasiveness, paralleled by loss of E-cadherin protein expression and increase in non-phosphorylated (nuclear) β-catenin expression, suggesting that SRF promotes liver metastasis through its action on membrane E-cadherin and β-catenin[32]. The oncogenic potential of SRF overexpression in the liver was further confirmed a few weeks ago by Farra and co-workers. Building on recent advances in the field mentioned above, they decided to test the effectiveness of SRF depletion in highly and poorly differentiated hepatocellular carcinoma (HCC) cell lines[33]. Their studies, which highlight differences in response to SRF depletion among different grades, also support a therapeutic role for SRF depletion against HCC, for which there are currently no effective treatment options[33].

Pancreas

The importance of SRF to the early phase of liver regeneration is well established by the studies above, however, they also noticed that the liver without SRF can still

Table 2 Tools and models available to study SRF functions in GI

Tool	Purpose	Model
SRF in pcDNA3.1, His[6,21]	SRF over-expression	Gene therapy
SRF antisense sequence[21,33]	SRF down-regulation (siRNA)	Gene depletion
Srf Flex1 mice[35]	Conditional *in vivo* SRF deletion	Gene depletion
Srf loxP mice[30]	Conditional *in vivo* SRF deletion	Gene depletion
CreER T2 mice[36]	SMS tissue expression	CIPO
AlfpCre mice[31]	Hepatocyte tissue expression	Liver function
K5-Cre mice[37]	Squamous epithelial expression	Esophagus, foregut
Fabp/Cre mice[38]	Conditional gut tissue expression	Small/large intestine
H. pylori[23]	*H. pylori* infection in human cell lines (*in vitro*) or mice (*in vivo*)	*H. pylori* gastric diseases

develop. A similar situation was also observed in the pancreas. Miralles and co-workers[34] found that mice with conditional inactivation of SRF in the pancreas had normal development of both the exocrine and endocrine pancreas. However, after weaning, these mice developed profound morphological alterations of the exocrine pancreas, which were reminiscent of severe pancreatitis. In these mice, massive acinar injury and pro-inflammatory cytokines release led to complete destruction of the exocrine pancreas and its replacement by adipose tissue.

SRF-related tools and models

Over the last decade, work on SRF in GI tissues has been very productive not only in establishing that SRF plays very important roles in both normal and pathological processes, but also in generating good *in vivo* model systems and validated tools to further study the role of SRF in both normal development and function as well as in related pathologies of the GI tract. Here we provide a detailed list of SRF-related models and tools (Table 2), which will be very useful for further exploration of the role of SRF in the GI tract. These include His-tagged SRF cDNA in the pcDNA3.1 mammalian expression vector under the control of the cytomegalovirus (CMV) promoter[6,21] for SRF over-expression in cell culture and gene therapy *in vivo*; validated antisense SRF oligonucleotide sequences to knock down SRF protein expression[21,33]. Two different conditional SRF knockout mice[30,35], are also available to generate temporally and spatially controlled SRF deletion in any desired tissue through the use of tissue-specific Cre mice. Currently, these have been combined with SMS[36]- and hepatocyte[31]-specific Cre mice to generate the CIPO model[24] and the liver model[30] of SRF, respectively. However, they hold unlimited potential for selectively knocking out SRF in any desired tissue or subpopulation of the GI tract to further study the role of SRF in GI. For instance, crossing either the SRF knockout line to the previously described K5-Cre transgenic mice[37] would allow SRF deletion in the basal cell layers of various squamous epithelial cells, including esophagus and foregut. Moreover, the Gordon group also generated two different Cre model systems (Fabp), which allow for intestinal-specific deletion, which could also be temporally controlled in a doxycycline inducible manner by combining Fabp-rtTA and tetO-Cre with the desired gene knockout[38].

FUTURE DIRECTIONS IN GI

Results summarized here clearly show that SRF is an important factor in mediating both normal and pathological conditions in the GI tract and that different optimal expression levels are associated with its various functions, while deviations from those levels can result in more or less severe pathological conditions. The flip side of that is that SRF could also lend itself to favorable manipulation, if we only know what it is. While providing initial clues and identifying several factors involved in SRF-mediated functions (Table 1), the findings above still leave the door wide open for additional exciting work in these areas of research, with particular focus on the finer details of SRF signaling in the individual processes, which would also help us understand how and when SRF could be a good therapeutic target.

Role of SRF in gastrointestinal ulcers

For instance, we show that SRF is critical for mediating the wound healing process in both gastric[6] and esophageal[21] ulcers, primarily through its role in VEGF-induced angiogenesis in a Rho A/actin- and ERK pathway-dependent manner[21]. However, while both Rho A and ERK have been linked to SRF induction[6], little is known about how they may interact with each other, and more studies along those lines would be extremely helpful in defining the role of SRF in this process and its potential as a therapeutic agent.

Role of SRF in gastrointestinal motility disorders

Similarly, preliminary work by Angstenberger and Merickskay established a very useful model for studying CIPO and trying to find possible treatment options for this very serious condition. Insights for new avenues into this area of research can be found in the three models proposed by de Giorgio[26]. In addition, given the emerging central role of SRF in the contractile function of the intestine, findings in this area of research may also be useful for less severe but more common dysfunctions of the intestine, with wider applications.

Role of SRF in *H. pylori* and related pathologies

H. pylori infection appears to be another major new area of research for SRF function in GI pathogenesis, which definitely deserves more attention. Here too, Hirata[22] and

Rieder[23] outline initial important connections between SRF and *H. pylori* infection and premalignant transformation, however, more work is needed to fill in the gaps. For instance, SRF activation appears to be involved in a new mode of CagA-mediated infection, which, given its more virulent nature, deserves more attention. In addition, work from Rieder and co-workers provide a preliminary molecular framework to further study the signaling components involved in SRF-mediated metaplasia[23]. A better understanding of the signaling cascades downstream and upstream of SRF in this process would be very useful for both diagnosis and more informed development of therapeutic options. For this purpose, the different *H. pylori* strains optimized for either *in vivo* mouse studies or human cell culture studies[23] (Table 2) will be particularly useful.

Role of SRF in gastrointestinal cancers

Overall, several lines of evidence seem to point to a positive role of SRF in various GI cancers, such as its role in driving angiogenesis[21], in mediating *H. pylori* infection[22] and metaplasia[23], which is strongly associated with gastric cancer, and in promoting cell proliferation in HCC[33] and liver metastasis by weakening cell adhesion[32]. Findings by Patten and co-workers that an SRF variant is also over-expressed in colon cancer in response to a very prevalent colon cancer mutation[27] further highlight the potential importance of SRF in cancer. However, the fact that the variant is a known dominant negative form of SRF makes its role in cancer progression not as straight forward. This is particularly true given findings by Choi and co-workers, which clearly correlate SRF over-expression with colon cancer progression[32]. However, since Patten and co-workers never examined actual tumor tissues and, while SRFΔ5 expression was indeed elevated in response to K-ras activation, full-length SRF was also elevated and generally showed much stronger expression than SRFΔ5 itself, more work may be required to better understand the actual prevalence of this variant in colon cancer and, most importantly, its role in cancer progression. More solid evidence to support the conclusions by Patten and co-workers could have important implications for diagnosis, identifying the SRFΔ5 variant as a possible marker for colon cancer. Since it is much easier to routinely collect small biopsies from colon tissue than from some other tissues, this could potentially be an effective way for better risk assessment.

Role of SRF in normal and abnormal liver function

Recent findings reported here suggest an interesting role for SRF in liver function. While Sun and co-workers show that not enough SRF is bad for proper liver function and regeneration after injury[31], studies from Ferra and co-workers raise hopes for effective HCC therapy through SRF depletion[33]. Clearly, this is just the tip of the iceberg and more data is needed in this area of research, where SRF is once again taking a leading role.

CONCLUSION

The last decade has been very prolific in shifting the focus of SRF from its role in the myocardium to a central role in the gastrointestinal tract as well. As summarized here, SRF is critical for proper development and function of most GI tissues in what appears to be a dose-dependent manner, as changes in its expression pattern have been implicated in various GI pathologies from intestinal motility disorders to cancer. In addition, both SRF gene therapy and SRF antisense expression to either elevate or inhibit normal SRF expression have been shown to hold great promise as potential therapeutic agents to either promote ulcer healing or inhibit cancer-related angiogenesis, respectively. Therefore, while great advances have already been made in this field, more in depth studies are warranted to fully understand its various roles and optimal expression requirements in all these processes, with particular attention to the potential therapeutic efficacy of SRF gene therapy and antisense expression where modulation of SRF expression may prove beneficial. For instance, SRF gene therapy could promote wound healing and liver regeneration, while its antisense expression could be more beneficial in slowing down cancer progression, where its effect on VEGF-mediated angiogenesis may play a central role in its dual applications.

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EDITORIAL

Systematic review and meta-analysis of *Saccharomyces boulardii* in adult patients

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Abstract

This article reviews the evidence for efficacy and safety of *Saccharomyces boulardii* (*S. boulardii*) for various disease indications in adults based on the peer-reviewed, randomized clinical trials and pre-clinical studies from the published medical literature (Medline, Clinical Trial websites and meeting abstracts) between 1976 and 2009. For meta-analysis, only randomized, blinded controlled trials unrestricted by language were included. Pre-clinical studies, volunteer studies and uncontrolled studies were excluded from the review of efficacy and meta-analysis, but included in the systematic review. Of 31 randomized, placebo-controlled treatment arms in 27 trials (encompassing 5029 study patients), *S. boulardii* was found to be significantly efficacious and safe in 84% of those treatment arms. A meta-analysis found a significant therapeutic efficacy for *S. boulardii* in the prevention of antibiotic-associated diarrhea (AAD) (RR = 0.47, 95% CI: 0.35-0.63, $P < 0.001$). In adults, *S. boulardii* can be strongly recommended for the prevention of AAD and the traveler's diarrhea. Randomized trials also support the use of this yeast probiotic for prevention of enteral nutrition-related diarrhea and reduction of *Heliobacter pylori* treatment-related symptoms. *S. boulardii* shows promise for the prevention of *C. difficile* disease recurrences; treatment of irritable bowel syndrome, acute adult diarrhea, Crohn's disease, giardiasis, human immunodeficiency virus-related diarrhea; but more supporting evidence is recommended for these indications. The use of *S. boulardii* as a therapeutic probiotic is evidence-based for both efficacy and safety for several types of diarrhea.

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Key words: Probiotic; Diarrhea; *Saccharomyces boulardii*; Adult patients; Meta-analysis

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INTRODUCTION

Probiotics have become increasingly popular in the US and are rapidly reaching levels of use in Europe and Asia, which have a longer history of use compared with the US[1-3]. Probiotics are "live microorganisms, which when administered in adequate amounts, confer a health benefit on the host"[4,5]. In one survey, of 435 users of dietary supplements, 50% of women and 33% of men reported use of probiotics[6].

Probiotics are generally recommended to help strengthen host systems and assist in recovery from certain diseases. However, the general public and many health care providers are still confused about how best to utilize probiotics and which ones are effective for specific diseases. There are several challenges in choosing the appro-

priate probiotic; including the wide diversity of probiotic strains, quality control of commercially-available probiotic products and the degree of evidence-based trials for each disease and probiotic. The ability of an organism to be an effective probiotic has been found to be strain-specific and microbial organisms are defined by their genus, species and strain. For example, *Lactobacillus rhamnosus* (*L. rhamnosus*) GG is a specific bacterial strain, which has been shown to be an effective probiotic for several diarrheal diseases, but other strains within the *L. rhamnosus* species may not be an effective probiotic. Similarly, other species under the same genus (*Lactobacillus*) may not be effective probiotics[7]. To add to this confusion, probiotic products are available in diverse forms: capsules of freeze-dried or lyophilized cultures, heat-dried culture supernatants, mixed in diary food (such as yogurts, cheese, milks, or ice cream) or other food (kefir, chocolate, wafers)[8-10]. The variety of probiotic products are also regulated under different guidelines according to the ways of use (food, dietary supplement, over-the-counter use or prescription). Different standards of quality assurance also vary with the type of product, indication for use and country.

Despite these challenges, the awareness of probiotics as a therapeutic modality has increased dramatically and the frequency of peer-reviewed randomized clinical trials have kept abreast with the global interest in this innovative method of therapy. Numerous probiotic strains have been investigated for clinical efficacy, including multiple bacterial strains: *Lactobacilli*, *Bifidobacteria*, *Streptococci*, Clostridia and fungal strains: *Saccharomyces boulardii (S. boulardii), S. cerevisiae*, and *Monascus purpureus*[11-13]. This review examines evidences for Saccharomyces probiotics and focuses on *S. boulardii* for the efficacy and safety of different diseases in adult patients. The two goals of this study were: (1) to provide guidance for clinicians and patients on how to appropriately use *S. boulardii* as a therapeutic probiotic and (2) to review the evidence for efficacy and safety of *S. boulardii* for various disease indications in adults.

SEARCH STRATEGY FOR SYSTEMATIC REVIEW

PubMed, Medline and Google Scholar were searched for articles unrestricted by language from 1976 to 2009. Three on-line clinical trial registers were searched: Cochrane Central Register of Controlled Trials (www.cochrane.org), metaRegister of Controlled Trials (www.controlled-trials.com/mrct) and National Institutes of Health (www.clinicaltrials.gov). Secondary and hand searches of reference lists, other studies cross-indexed by authors, reviews, commentaries, books and meeting abstracts also were performed. Search terms included: *Saccharomyces boulardii*, probiotics, yeast, diarrhea, risk factors, randomized controlled trials, placebo-controlled, and associated author names. Search strategies were broad-based initially, then narrowed to the disease of interest to increase the search network[14].

META-ANALYSIS

If sufficient numbers of clinical trials (> 5) were found on one type of disease indication, a meta-analysis for the efficacy of *S. boulardii* was performed; otherwise a systematic review was done. The procedure for the meta-analysis followed standard meta-analysis methodology with clearly delineated parameters, *a priori* inclusion and exclusion criteria and standardized data extraction[15,16]. Abstracts of all citations and retrieved studies were reviewed and rated for inclusion. In some cases, only published abstracts from meetings were available. Published abstracts from meetings were included to lessen the potential for publication bias due to failure to publish negative findings. Information on study design, methods, interventions, outcomes, adverse effects and treatments was extracted from each article using a standardized extraction table. When necessary, authors were contacted for data not reported in the original article.

The primary objective of the meta-analysis was to determine the overall efficacy of *S. boulardii* within one specific type of disease indications (antibiotic-associated diarrhea, *Clostridium difficile* disease, traveller's diarrhea, irritable bowel syndrome, inflammatory bowel disease or other types of diarrhea). Inclusion criteria were: randomized, controlled, blinded efficacy trials in humans published as full articles or meeting abstracts in peer-reviewed journals. Exclusion criteria included: use of other strains of Saccharomyces (such as *Saccharomyces cerevisiae*) or other strains of probiotics, pre-clinical studies, safety studies, case reports or case series, phase I studies in volunteers, reviews, duplicate reports, trials of unspecified treatments, uncontrolled studies, prebiotic treatments only (no living organisms) or insufficient data in article.

To estimate pooled relative risks across studies, heterogeneity between and within trials was evaluated using the Cochrane Q and I^2 tests[17]. Relative risks (RR) and 95% confidence intervals (CI) were calculated using the Mantel-Haenszel method. The relative risks of responding to *S. boulardii* therapy were pooled using a random-effect model if significant heterogeneity was found or a fixed-effect model if the studies were homogenous. P values less than 0.05 were considered significant. Analyses were performed using Stata software version 9.2 (Stata Corporation, College Station, Texas). A funnel scatterplot and Begg's test were used to assess publication bias[18].

HISTORY

Saccharomyces cerevisiae strains have a long history of use in baking and brewing preparation, but have only infrequently been investigated for probiotic properties[19,20]. Another closely related strain, *S. boulardii*, was discovered by a French microbiologist, Henri Boulard in 1920 when he was in IndoChina searching for new strains of yeast that could be used in fermenting processes. He was a visitor during a cholera outbreak and noticed that some people who did not develop cholera were drinking a special tea. This tea was

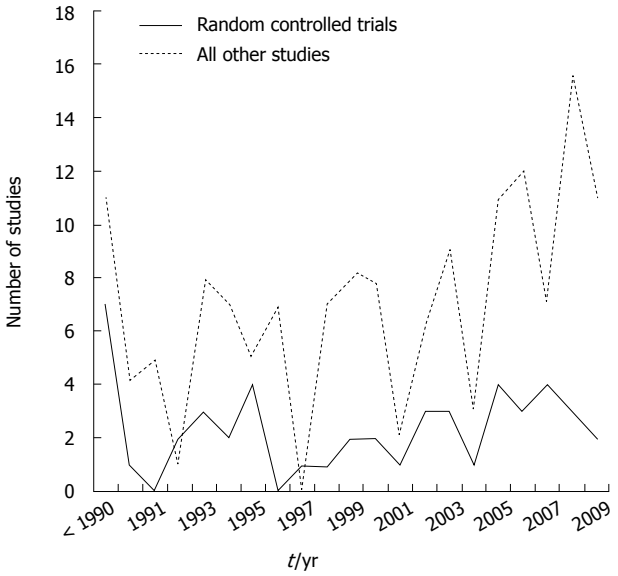


Figure 1 Frequency of peer-reviewed publications on *Saccharomyces boulardii* from 1976 to 2009.

made by taking the outer skin from a tropical fruit (lychee and mangosteens) and cooking them down to make tea. He succeeded in isolating the agent responsible. It was a special strain of yeast he named "*Saccharomyces boulardii*". The patent for this yeast was bought by Laboratories Biocodex in 1947, which began researching and manufacturing protocols. As shown in Figure 1, interest in this strain has increased, as reflected by the increasing number of scientific publications, encompassing both pre-clinical papers in mechanisms of action, animal models of efficacy, pharmacokinetics, early studies in safety and dose-ranging. In addition, there are currently 53 randomized controlled clinical trials, encompassing 8475 subjects, investigating the safety and efficacy of *S. boulardii* in pediatric and adult patients spanning several disease indications, of which 43 (81%) found significant efficacy for *S. boulardii*. As a recent review article on the efficacy of *S. boulardii* was published for pediatric indications[21], this study will focus on *S. boulardii* use in adult patients. There have been 27 randomized controlled trials, with 31 treatment arms testing *S. boulardii* in adult patients for a variety of diseases and most (84%) found a significantly protective efficacy of this probiotic. There have been only a few randomized clinical trials using other strains of Saccharomyces probiotics, which will be discussed later in this paper.

TAXONOMY

Probiotics may be either bacterial or yeast microbes. Yeast probiotics, such as *S. boulardii*, are different from bacterial probiotics (different physiologic structures, large in size, do not acquire antibiotic-resistant genes and are not affected by antibiotics)[12]. Not only is there a confusing array of probiotic products on the global market, but the taxonomy of Saccharomyces strains has been debated[22]. One strain has received considerable discussion about its valid nomenclature[23,24]. It was originally named *S. boulardii* in the 1950's. Advances in typing methods opened a debate as to whether this strain should be reclassified as a strain of *S. cerevisiae* or remain a separate species. Early work using PCR electrophoretic karotyping or rRNA sequencing methods reported that *S. boulardii* was indistinguishable from other strains of *S. cerevisiae*[24,25]. Newer metabolomic tools (microsatellite polymorphism analysis and retrotransposon hybridization analyses) show that *S. boulardii* has a unique clustering different from other strains of *S. cerevisiae*[26-28]. In addition, *S. boulardii* differs from other strains of *S. cerevisiae* by several metabolic and genetic characteristics[29,30]. *S. boulardii* persists longer in gnotobiotic mice models (10 d) compared with rapid clearance of other strains of *S. cerevisiae* (< 1 d)[31]. *S. boulardii* is also different physiologically and metabolically and its optimum growth temperature is 37℃ and it is resistant to low pH and is tolerant to bile acids; whereas other strains of *S. cerevisiae* prefer cooler temperatures (30-33℃) and do not survive well in acid pH ranges[32-34]. *S. boulardii* can be distinguished from other strains of *S. cerevisiae* by advanced typing methods, by differences in metabolism and physiology and by the ability to have anti-pathogen effects (as discussed in the mechanisms of action section).

GUIDELINES FOR CHOOSING APPROPRIATE PROBIOTICS

Challenges in providing guidance to patients regarding the appropriate choice of probiotic include the wide diversity of available products on the market, variances in quality control, stability and formulations in those products, and the requirement to match the type of probiotic with the disease indication. The efficacy of probiotics have been shown to be both strain-specific and disease-specific[11,35,36].

Product to product variation

There are many different Saccharomyces products available commercially. Table 1 lists some examples of products that contain *S. boulardii*, sold as probiotics either as lyophilized or heat-dried powders in capsules, or as one of several strains in a probiotic mixture in capsules or in liquid beverages[12,13,21,37-66]. The quality of these products from different sources have been found to vary. Choosing a probiotic product from a manufacturer with a regulated quality control program is a sound policy. Unfortunately, many of the products available commercially may lack regulated quality control programs. Studies of other probiotics have found a wide diversity in both quality and contamination in products available on the Internet[67]. Marcobal *et al*[68] tested 14 commercial probiotics in the US and found 93% were incorrectly labeled (57% had contaminants and 36% did not list strains on the label). Masco *et al*[69] tested 58 different probiotic products from Europe, UK, Asia, Japan and Canada and found only 38% had the dose stated on the label and 29% did not contain strains listed on the label. Not all products were found to have high standards of manufacture and quality control, as only some conform to Good Manufacturing Practices.

Table 1 Examples of commercially available probiotics containing "*Saccharomyces boulardii*"

Product name	Probiotic strain	Manufacturer	Stable at room temp[1]	Facility certified	Strain confirmed	Colony forming unit (cfu) per mg capsule or mL	Original strain-specific studies	Degree of clinical evidence[2]
Single strain								
Florastor® (US) Perenterol® (Germany) Reflor® (Turkey) Ultra-Levure® (Asia)[3]	*S. boulardii* lyo	Biocodex (France)	Yes	EU GMP	Microsatellite sequency polymorphism [28,29]	5×10^9/ 250 mg	Multiple [37-62]	A
SB+MOS	*S. boulardii*	Jarrow Formulas (Los Angeles, CA) and Gnosis (Italy)	No	EU GMP	Not stated	1.5×10^9	One study[63]	C
Saccharomyces boulardii	*S. boulardii*	Kirkman (Oregon)	No	GMP	DNA fingerprint	3×10^9/ 150 mg	None	F
Saccharomyces boulardii	*S. boulardii*	Allergy Research Group (CA)/NutriCology (CA)	No	GMP	DNA fingerprint	3×10^9/ 150 mg	None	F
Saccharomyces boulardii	*S. boulardii*	Klaire Labs (Reno Nevada)	No	GMP ISO 9001	Ribotyping	3×10^9/ 150 mg	None	F
In mixtures of probiotics								
Protecflor® (Canada) Erce Flora® (Belgium)	*S. cerevisiae boulardii* CNCM I-1077) + *L. rhamnosus* + *L. acidophilus* + Bifido. strain	Institut Rosell-Lallemand (Quebec, Canada)	Not stated	Canadian GMP	Not stated	10^9/2 mL	Two studies [64,65]	D
MitoMix®	*S. boulardii* and *Pediococcus acidilactici*	Imagilin Technology (Maryland)	Yes	Not stated	Not stated	2.3×10^9/ capsule	One study[66]	D
Primal Defense™	*S. boulardii* with 13 other strains[4]	Garden of Life (FL)	Yes	Not stated	No (only certified "organic")	Total 1×10^9 per 410 mg	None	F
Pro-Bio Defense™	*S. boulardii* + 7 other strains[5]	Kirkman (OR)	No	GMP	DNA fingerprint	Total: 2×10^{10}/cap, no data on SB cfu	None	F
ABX Support™	*S. boulardii* + *L. rhamnosus* + *Bifido. bifidum* + *Bifido. breve*	Klaire Labs (NEV)	No	GMP ISO 9001	Ribotyping	5×10^9/ 300 mg	None	F
Kombucha fermented tea	*S. boulardii* + *L. bacterium* + blue-green algae	Millennium Products, Inc.	No	No	Not stated	1×10^9 per 16 oz.	None	F

[1]Products not stable at room temperature require refrigeration, probably heat-dried. Products stable at room temperature are typically packaged in blister packs and are lyophilized; [2]Degree of evidence: A = at least two randomized, controlled clinical trials in patients; B = one randomized controlled clinical trial in patients, C = case reports, uncontrolled randomized trials or open safety/kinetic studies in patients or volunteers, D = *in vivo* animal model or *in vitro* studies only, E = expert opinion only, F = no direct evidence; [3]A few examples, Biocodex has over 40 brand names worldwide; [4]Other strains in Primal Defense include: 11 strains of *Lactobacilli* (*L. acidophilus, L. bulgaricus, L. lactis, L. plantarum, L. casei, L. lactis, L. leichmannii, L. brevis, L. caucasicus, L. fermenti*, *L. helveticus* and 1 strain each: *Bifidobacterium bifidum, Bacillus subtilis, Bacillus lichenformis*); [5]Other strains in Pro-Bio Defense include: 5 strains of *Lactobacilli* (*L. plantarum, L. rhamnosus, L. acidophilus, L. casei, L. bulgaricus*) and *Bifidobacterium lactis* and *Streptococcus thermophilus*. GMP: Good manufacturing practices.

Although most products state they contain at least 1×10^9 *S. boulardii*/mg, independent assays have determined 50% of the products contained a dose less than on the label. In one study comparing six *S. boulardii* products, all had identical PCR typing profiles, but only 50% [Floratil (Merck), Flomicin (NeoChemical), and Florazin (Herald's)], had the same concentration identified on their label. One product [Lactipan (Sigma Pharm)] had 2×10^4 fewer *S. boulardii* than stated on its label. One product [Floratil (Merck)], had the highest concentration (1×10^9/100 mL) and maintained high levels (9.5×10^8) six months later[70]. Even if the label states it contains *S. boulardii*, a variation in efficacy may occur due to lower than stated dose or inaccurate strain composition. Four *S. boulardii* products were tested (along with one *S. cerevisiae* product) in Brazil. Only two (50%) of the *S. boulardii* products were protective in a *Salmonella typhimurum* mouse model (two *S. boulardii* products were ineffective, as well as the one *S. cerevisiae* product)[71]. Without access to specific quality control assays for commercially available probiotic products, the choice of a high quality product can be difficult. One method of selecting a probiotic product is to find a product in which the manufacturing company has sponsored original clinical trials, as this indicates a degree of commitment that may not be present in companies that do not sponsor original research. As shown in Table 1, only a few *S. boulardii* probiotic products are supported by original research. Although at present, most probiotic clinical trials are sponsored by private companies, as more national funding becomes available, this situation may change.

Stability

How the probiotic product is manufactured may significantly affect its potency over time (shelf-life). Probiotics may be available as lyophilized preparations, heat-dried preparations or contained in diary or drink food products.

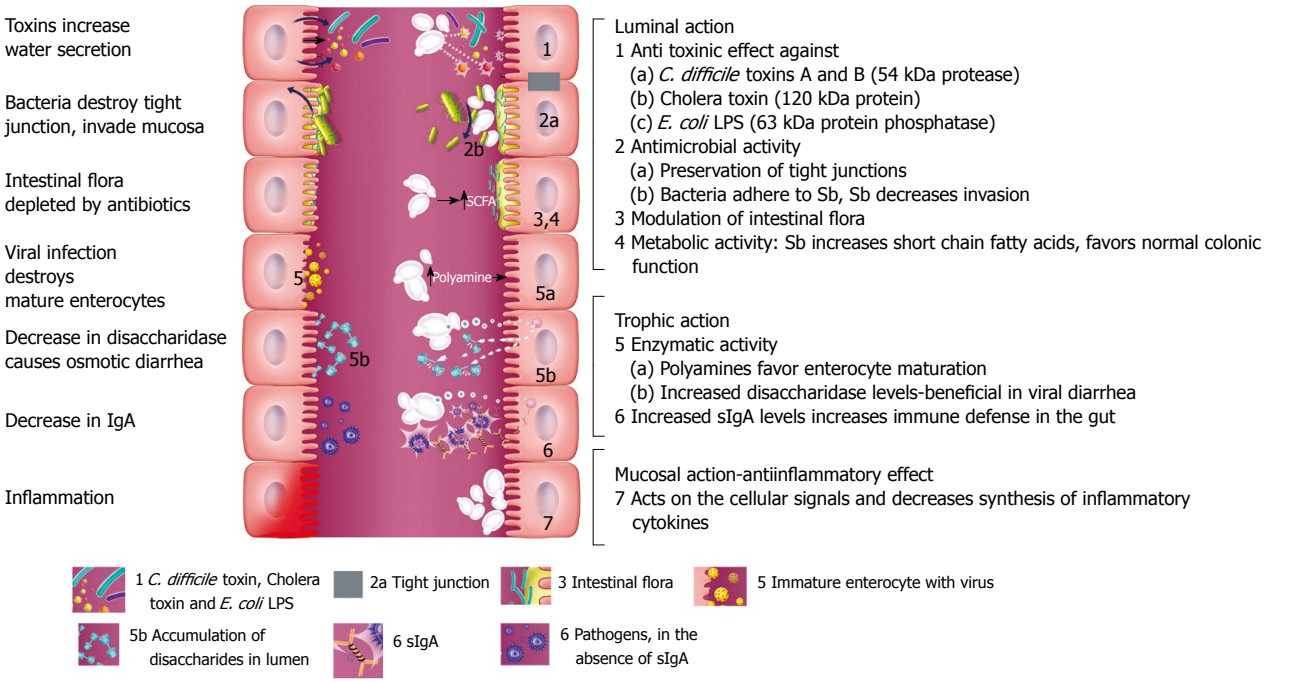


Figure 2 Schematic of intestinal tract, illustrating the different potential mechanisms of action of *Saccharomyces boulardii* (Sb). On the left, effects of different pathogenic microbes are depicted. On the right, seven different protective effects of *S. boulardii* are depicted. Within the lumin of the intestine, *S. boulardii* may degrade toxins of pathogens, interfere with pathogenic adherence, modulate normal microbiota and preserve normal intestinal physiology. *S. boulardii* may also indirectly restore normal short chain fatty acid (SCFA) balance. *S. boulardii* may also increase secretory IgA (sIgA) levels or act as an immune regulator by influencing cytokine levels.

S. boulardii is usually available in capsules of either lyophilized or heat-dried preparations. Heat-dried capsule products may be identified by their labels, which usually state that the products should be refrigerated after opening and they lose their potency rapidly. Lyophilized preparations of *S. boulardii* are stable over one year at room temperature, as long as it is protected from moisture[72]. Lyophilized products are stable at room temperature and have the advantage of portability and convenience and maintain high viability counts over prolonged periods. Heat-dried preparations are not stable at room temperature and must be refrigerated. A study of four *S. boulardii* products in Germany found a lyophilized product [Perenterol Forte (Thiemann)] outperformed three heat-killed *S. boulardii* preparations in terms of higher viable cells and quicker start-up times[73].

Single strain or probiotic "cocktails"

As shown in this review, all the randomized controlled trials using *S. boulardii* probiotics have utilized a single strain preparation. Although mixtures of probiotics, which may contain *S. boulardii*, are available on the market, no randomized controlled trials have been done showing that these mixtures are superior to the single stain preparations. Pre-clinical studies in animal models have found promising results in a probiotic mixture (*L. rhamnosus*, *L. acidophilus, Bifidobacterium* and *S. boulardii*) for reducing *E. coli* diarrhea in rats, but no human clinical trials have been performed with this mixture[65]. Another potential limitation to probiotic mixtures is antagonism between the different probiotic strains and conflicting mechanisms of actions that may tend to attenuate the therapeutic responses of the probiotic strains[74].

Mechanisms of action

An advantage of probiotics is that they are living organisms incorporating a delivery system (most probiotics survive to the target organ) bringing an arsenal of anti-pathogenic strategies into play. *S. boulardii* has several different types of mechanisms of action (Figure 2) which may be classified into three main areas: luminal action, trophic action and mucosal-anti-inflammatory signaling effects[8,75-77]. Within the intestinal lumen, *S. boulardii* may interfere with pathogenic toxins, preserve cellular physiology, interfere with pathogen attachment, interact with normal microbiota or assist in reestablishing short chain fatty acid levels. *S. boulardii* also may act as an immune regulator, both within the lumen and systemically.

Anti-toxin effects: *S. boulardii* may interfere with pathogenesis within the intestinal lumen by several mechanisms: either by blocking pathogen toxin receptor sites[78], or acting as a decoy receptor for the pathogenic toxin[79] or by direct destruction of the pathogenic toxin. Castagliuolo *et al*[80] found a 54 kDa serine protease produced by *S. boulardii* directly degrades *C. difficile* toxin A and B. The efficacy of other strains of Saccharomyces has also been investigated. Only *S. boulardii* produces a protease capable of degrading *Clostridium difficile* toxins and receptors sites on the enterocyte cell surface, unlike other strains of Saccharomyces[78,81]. Buts *et al*[82] found a 63 kDa phosphatase produced by *S. boulardii* destroys the endotoxin of pathogenic *E. coli*. Several investigators showed that *S. boulardii* could reduce the effects of cholera toxin and this may be due to a 120 kDa protein produced by *S. boulardii*[83,84].

Antimicrobial activity: *S. boulardii* is capable of directly or indirectly interfering with intestinal pathogens. *S. boulardii* may directly inhibit the growth of pathogens (such as *Candida albicans*, *Salmonella typhimurum*, *Yersinia enterocolitium*, *Aeromonas hemolysin*[43,85,86]). In animal models testing for the ability to inhibit pathogen growth, several studies using non-*S. boulardii* strains of *S. cerevisiae* did not find any effect unlike the protective effects of *S. boulardii*[31,70]. A few studies have tested other strains of *S. cerevisiae* and found promising results. Brandao *et al*[79] tested *S. cerevisiae* strain W303 in rats and found less histologic damage due to cholera toxin compared with saline controls, but no clinical trials have been done using this strain. *S. boulardii* may also act by enhancing the integrity of the tight junction between enterocytes, thus preserving intestinal integrity and function[87,88]. Wu *et al*[88] found less crypt hyperplasia and cell damage in a *Citrobacter rodentium*-induced mice model of colitis when mice were treated with 1×10^9 *S. boulardii* per day for 7 d. Garcia Vilela *et al*[62] found decreased intestinal permeability when patients with Crohn's disease were given *S. boulardii* (1.6×10^9/d for 4 mo) compared with placebo. *S. boulardii* has also been shown to reduce the translocation of pathogens in rat and pig animal models[64,89,90]. *S. boulardii* can also interfere with pathogenic attachment to intestinal receptor sites[88,91,92]. Gedek *et al*[93] also found that *S. boulardii* acts as a decoy by causing EPEC cells to directly bind to the surface of *S. boulardii* cells rather than enterocytes.

Cross-talk with normal microbiota: Newer techniques, including metagenomics and PCR probes have documented that a typical human may carry over 40 000 bacterial species in the collective intestinal microbiome[94]. The normal intestinal flora has many functions, including digestion of food, but the one that is most germane for this discussion is called "colonization resistance"[77,95,96]. This involves the interaction of many bacterial microflora and results in a barrier effect against colonization of pathogenic organisms. Normal microflora may act by competitive exclusion of nutrients or attachment sites, produce bacteriocins, or produce enzymes detrimental to pathogenic growth. Factors that disrupt this protective barrier, for example antibiotic use or surgery, results in host susceptibility to pathogen colonization until such time as the normal microflora can become re-established. Typically, it takes six to eight weeks for normal microbiota to recover after antibiotic exposure or disease resolution[97]. Probiotics are uniquely qualified to fit into this window of susceptibility and may act as surrogate normal microflora until recovery is achieved. *S. boulardii* has no effect on normal microbiota in healthy human controls[98,99]. In contrast, when *S. boulardii* is given to antibiotic-shocked mice or patients with diarrhea, normal microbiota is re-established rapidly[99,100].

Restoration of metabolic activities: *S. boulardii* has been shown to be able to increase short chain fatty acids (SCFA), which are depressed during disease, indicating altered colonic fermentation[98,101,102].

Trophic effects: *S. boulardii* can reduce mucositis[103], restore fluid transport pathways[84,101,104], stimulate protein and energy production[105], or act through a trophic effect by releasing spermine and spermidine or other brush border enzymes that aid in the maturation of enterocytes[106,107].

Immune response: *S. boulardii* may also regulate immune responses, either acting as an immune stimulant or by reducing pro-inflammatory responses. *S. boulardii* may cause an increase in secretory IgA levels in the intestine[56,108-110]. It has also been found associated with higher levels of serum IgG to *C. difficile* toxins A and B[111]. *S. boulardii* may also interfere with NF-κB-mediated signal transduction pathways, which stimulate pro-inflammatory cytokine production[76,112,113]. Chen *et al*[114] found that *S. boulardii* blocks activation of ERK1/2 and MAP kinases, which typically stimulate IL-8 production and cell necrosis in mice ileal loop models and in *in vitro* models. *S. boulardii* has also been shown to cause the trapping of T helper cells into mesenteric lymph nodes, thereby reducing inflammation[115].

Properties of *S. boulardii*

To be an effective probiotic, it must survive passage to its target organ (most commonly the colon). Organisms need to survive at body temperature (37℃), be resistant to stomach acids and bile acids, and exist in the competitive milieu of the intestinal tract. Probiotic strains of Saccharomyces have been shown to have these abilities. Although the optimal temperature for most strains of Saccharomyces range from 22-30℃, *S. boulardii* survives best at 37℃, giving it a unique advantage of being one of the few yeasts that do best at human body temperatures.

Survival to target sites: Although much of the oral dose is destroyed (usually stool levels are 100-1000 times lower than the oral dose), surviving oral doses have been found to be effective (usually at levels over 10^8 organisms/g stool)[116]. *S. boulardii* is resistant to antibacterial agents and survives gastric acidity[34,117]. In human volunteers, the concentration in the colon was found to be dose-dependent[118]. When *S. boulardii* was given to healthy volunteers at doses typically used therapeutically ($1\text{-}2 \times 10^{10}$/d), colonic levels were 2×10^8/g stool[118]. Few clinical trials using probiotics have documented the level of organisms present in the terminal site (colonic lumen in their study subjects). However, in one trial of patients with recurrent *C. difficile* infection (CDI) given *S. boulardii* (2×10^{10}/d) for 28 d, patients who had a subsequent CDI recurrence were found to have significantly lower numbers of *S. boulardii* (2×10^4/g stool) compared with those without recurrence (1×10^6/g stool)[119].

Pharmocokinetics: *S. boulardii*, when given orally, achieves steady-state concentrations within three days and is cleared within 3-5 d after it is discontinued[34,119,120]. Blehaut *et al*[120] gave eight healthy human volunteers *S. boulardii* (oral dose

of 5×10^9) for six days and followed them for time-to-clearance. They determined *S. boulardii* has half-life of 6 h, fecal steady-state concentrations (2×10^7/g) were reached by day 3 and the yeast was cleared after four days after administration. Klein *et al*[118] confirmed these findings in their studies of human volunteers and also found *S. boulardii* levels were 23 times higher in volunteers with disturbed intestinal microbiota (due to ampicillin exposure) than volunteers who were not given ampicillin. Elmer *et al*[121] found that some types of fiber (psyllium) increased *S. boulardii* levels by 22%, while other types of fiber (pectin) showed no effect.

CLINICAL EFFICACY: ACUTE DISEASES

S. boulardii probiotics have been tested for clinical efficacy in several types of acute diseases including antibiotic-associated diarrhea, *C. difficile* infections, *Helicobacter pylori* (*H. pylori*) disease, acute adult diarrhea, enteral nutrition-related diarrhea, and traveler's diarrhea.

Antibiotic-associated diarrhea

Epidemiology of antibiotic-associated diarrhea: The reported incidence of antibiotic-associated diarrhea (AAD) ranges from 12/100 000 to 34/100[122] depending upon the type of antibiotic, host factors (age, health status, *etc.*), etiology, hospitalization status and presence of a nosocomial outbreak. The highest frequency of AAD is found (26%-60%) during healthcare associated outbreaks, when susceptible patients are clustered by time, exposure and proximity[123]. Healthcare associated (hospital, long-term care facilities, nursing homes) outbreaks of AAD are to be expected because inciting agents (antibiotics), infectious agents and a susceptible patient population are intermixed. Historically, most cases of AAD were reported in hospitalized patients[122]. More recently, although AAD still occurs in hospital settings, rates of 6-33/100 have been reported in outpatient populations[124,125] and lower rates (12/100 000 to 14/100) in non-hospitalized adults[126,127]. Lower rates observed in outpatients may be due to their generally higher health status compared with hospitalized patients and also to the lack of exposure to nosocomial pathogens that commonly contaminate hospital environments. Higher incidences are still found in healthcare associated pediatric and adult patients (ranging from 5-34/100 patients)[128-130].

The clinical presentation of AAD may be mild (uncomplicated diarrhea) or more severe (colitis), or result in toxic megacolon or death[122]. The onset of AAD may occur while the patient is on antibiotics, but delayed-onset AAD is more common[122]. In addition, the onset of AAD may vary according to the type of antibiotic. Elmer *et al*[131] found a variable time of onset using the hamster model of AAD and different types of antibiotics. Early onset of AAD was associated with clindamycin, amoxicillin and ampicillin, while delayed-onset AAD was associated with erythromycin, ciprofloxacin and clarithromycin. Consequences of AAD may result in extended hospital stays, increased medical care costs and increased diagnostic procedures[122,132]. Prevention of AAD has rested on discontinuing the inciting antibiotic or switching to an antibiotic with a narrower spectrum of action, but there are no other current effective preventive measures for AAD.

Efficacy evidence for AAD: There have been ten randomized controlled trials in adults using *S. boulardii* for the prevention of AAD (Table 2)[37,42,44,54,55,58,133-136]. These studies have used a variety of daily doses, durations of treatment, length of follow-up and types of patient population. Of the ten controlled trials, 8 (80%) showed significant efficacy for the prevention of AAD compared with controls. One of the earliest trials by Adam *et al*[37], randomized 388 hospitalized patients to 200 mg of *S. boulardii* [4×10^9 cfu (colony forming units)/d] or placebo for seven days and found a significant reduction in AAD in the *S. boulardii group* (4.5%) compared with the controls (17.5%, $P < 0.05$). The protective effect of *S. boulardii* was confirmed in later studies. Monteiro *et al*[55] enrolled 240 patients receiving oral antibiotics and found significantly fewer patients randomized to *S. boulardii* developed AAD (15.7%) compared with placebo (27.7%, $P < 0.05$). McFarland *et al*[54] enrolled 193 hospitalized patients receiving beta-lactam antibiotics and randomized them to either 1 g of *S. boulardii* or placebo for the duration of the antibiotic treatment plus three additional days. Significantly fewer patients developed AAD while on the beta-lactam antibiotics or in the seven week follow-up period when given *S. boulardii* (7.2%, $P < 0.05$) compared with 14.6% of those given placebo. This study confirmed an earlier study by Surawicz *et al*[58] of 180 hospitalized adults randomized to either *S. boulardii* or placebo for the duration of their antibiotic treatment plus an additional two weeks. Of the 23% patients who were contacted 2-3 wk after the study, only one patient given placebo developed delayed-onset AAD. Only 9.5% of those randomized to *S. boulardii* developed AAD compared with 21.8% of those on placebo ($P < 0.05$). Can *et al*[135] enrolled 151 adult inpatients receiving various types of antibiotics, who were then randomized to *S. boulardii* or placebo for the duration of the antibiotic treatment. Significantly fewer patients (1.4%, $P < 0.05$) of those given *S. boulardii* developed AAD compared with the control group (9.0%). Three trials have been in conducted in patients with *H. pylori* infections receiving triple therapy (usually two antibiotics and an acid suppressor), which is associated with a high rate of AAD development. Duman *et al*[44] enrolled 389 patients with peptic ulcer or non-ulcer dyspepsia in nine hospitals in Turkey to observe if lower rates of side-effects could be achieved. This study compared 204 patients who received 1 g of *S. boulardii* (2×10^{10}/d for 2 wk) and triple therapy with 185 controls, who received only the triple therapy. Of the 389 patients, 376 completed the treatment phase and the four-week follow-up. Significantly fewer patients given *S. boulardii* (6.9%) developed AAD compared with the control group (15.6%, $P = 0.007$). Two other randomized controlled trials in adult patients receiving triple therapy for *H. pylori* infections were conducted and both showed a significant reduction in AAD for those treated with *S. boulardii*. Cremonini *et al*[134] randomized 85 *H. pylori* carriers to placebo, *L. rhamnosus* GG, *S. boulardii* or a mix of

Table 2 Randomized, controlled trials for the prevention of antibiotic associated diarrhea (AAD) using *S. boulardii*

Ref.	Treatment groups	Population	Daily dose: cfu/d (mg/d)	Duration (d)	Follow-up (wk)	AAD in *S. boularii* (%)	AAD in controls (%)
Adam *et al*[37]	*S. boulardii* *vs* placebo	388 hospitalized adults, multisite	4×10^9 (200 mg)	7	0	9/199 (4.5)[a]	33/189 (17.5)
Monteiro *et al*[55]	*S. boulardii* *vs* placebo	240 adults, Portugal	(nr) 4 caps/d	6	0	19/121 (15.7)[a]	33/119 (27.7)
Surawicz *et al*[58]	*S. boulardii* *vs* placebo	180 hospitalized adults at one hospital, Washington	2×10^{10} (1000 mg)	abx + 14 d	2-3	11/116 (9.5)[a]	14/64 (21.8)
McFarland *et al*[54]	*S. boulardii* *vs* placebo	193 hospitalized adults (1 or more beta-lactam antibiotics), 4 hospitals	2×10^{10} (1000 mg)	abx + 3 d	7	7/97 (7.2)[a]	14/96 (14.6)
Lewis *et al*[133]	*S. boulardii* *vs* placebo	72 enrolled, 69 done; elderly patients	4.5×10^9 (226 mg)	14	0	7/33 (21)	5/36 (13.9)
Cremonini *et al*[134]	*S. boulardii* *vs* placebo	43 *H. pylori* + adults on triple therapy	5×10^9 (nr)	14	2	1/22 (5)[a]	6/21 (30)
Duman *et al*[44]	*S. boulardii* *vs* placebo	389 adults in Turkey with *H. pylori* + peptic ulcers, all received triple therapy	2×10^{10} (1000 mg)	14	4	14/204 (6.9)[a]	28/180 (15.6)
Can *et al*[135]	*S. boulardii* *vs* placebo	151 adult inpatients	1×10^{10} (500 mg)	abx	4	1/73 (1.4)[a]	7/78 (9.0)
Cindoruk *et al*[42]	*S. boulardii* *vs* placebo	124 adults with *H. pylori* + dyspepsia	2×10^{10} (1000 mg)	14	6	9/62 (14.5)[a]	19/62 (30.6)
Bravo *et al*[136]	*S. boulardii* *vs* placebo	89 adult outpatients on amoxicillin	1×10^{10} (500 mg)	12	9	3/41 (7.3)	5/45 (11.1)

[a]$P < 0.05$, probiotic *vs* controls. cfu: Colony forming unit; nr: Not reported; abx: Antibiotics.

L. acidophilus and *Bifidobacterium lactis* and found significantly fewer patients developed AAD in only the *S. boulardii* group (5%, $P = 0.05$) compared with placebo (30%). Cindoruk *et al*[42] also found a significant reduction in AAD in adult patients treated with triple therapy for *H. pylori* infections for those randomized to *S. boulardii* (14.5%, $P < 0.05$) compared with placebo (30.6%). None of these trials reported any adverse reactions associated with *S. boulardii*.

It is important to have a sufficiently long follow-up period (usually 4-6 wk after cessation of antibiotics) to capture delayed-onset AAD. Several studies have shown that AAD may occur in patients on antibiotics, but AAD may also be delayed for up to two months (in up to 38% of patients) after antibiotics are discontinued[54,129]. Most studies of AAD followed patients for 4-6 wk after antibiotic treatment to document delayed-onset AAD[42,44,135]. In contrast, Lewis *et al*[133] found no significant reduction in AAD in a study of 69 elderly patients randomized to 226 mg of *S. boulardii* (4.5×10^9 cfu/d) or placebo for 14 d. This failure may have been due to a flawed study design, in that patients were only followed while on antibiotics and no follow-up was done after antibiotics were discontinued. The study by Lewis *et al*[133] therefore, may have missed a significant number of AAD cases due to a too short follow-up period. Short or no follow-up after antibiotic exposure may explain why some studies[133,136] did not find a significant protective effect of *S. boulardii*[137]. No adverse reactions associated with *S. boulardii* were noted in any of these studies. No other strains of Saccharomyces have been found to be effective to prevent AAD.

Meta-analyses for AAD: The literature search yielded 322 unique citations on *S. boulardii*, of which 278 were excluded: non-*S. boulardii* studies ($n = 19$), reviews ($n = 84$), pre-clinical studies including animal models of efficacy, mechanism of action, *in vitro* assays or strain characterization ($n = 130$), case reports or case series ($n = 15$), and phase Ⅱ safety or pharmacokinetic studies ($n = 30$). Of the 44 randomized controlled trials with *S. boulardii* screened, 34 were excluded (pediatric populations, $n = 17$; or non-AAD indications in adults, $n = 17$) and 10 were included (AAD in adult populations). As significant heterogeneity was found among studies, a random effect model was used. The random effect model gave a $\chi^2 = 10.8$, $P = 0.29$, indicating that the heterogeneity was well controlled. A meta-analysis of the ten randomized, controlled trials in adults presented in this review found that *S. boulardii* was significantly protective for AAD (Figure 3), with a pooled relative risk of 0.47 (95% confidence interval 0.35-0.63, $P < 0.001$). Begg's test did not find any significant publication bias ($P = 0.93$), nor did the funnel plot (data not shown). The number needed-to-treat (NTT) to prevent one case of AAD was 10.2.

In the established medical literature, meta-analysis has been used to combine different probiotic strains and studies to obtain a pooled estimate of the efficacy of probiotics for the prevention of AAD. A note of caution, however, while most meta-analyses have concluded that probiotics are effective for preventing AAD[123,138], it is inappropriate to conclude that all probiotic strains are effective. The effectiveness of probiotics is strain-specific and disease-specific, so each probiotic strain must be linked to the disease. Most probiotic meta-analyses have focused on one type of disease indication with a variety of probiotic strains (e.g. antibiotic associated diarrhea)[123,138,139]. When there are sufficient numbers of clinical trials, a meta-analysis limited to one disease indication and one type of probiotic strain may reduce the heterogeneity of studies[140]. Szajewska *et al*[141] limited her meta-analysis to randomized controlled trials of one type of probiotic (*S. boulardii*) in adults and children.

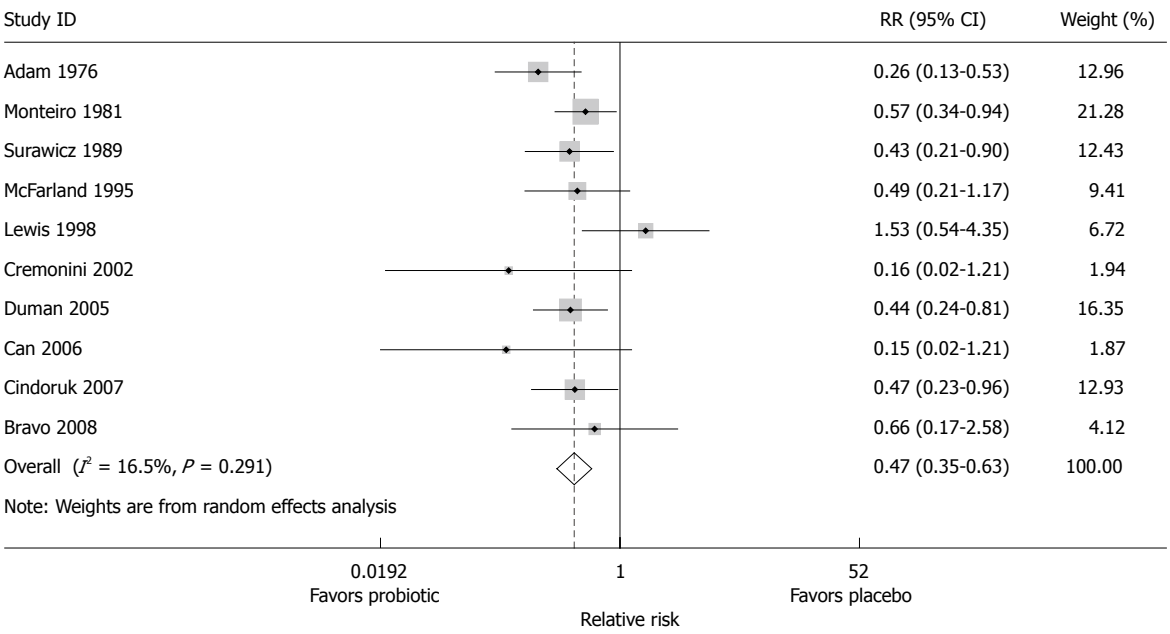


Figure 3 Forest plot of meta-analysis of ten randomized controlled trials measuring protective effect after treatment with *S. boulardii* or placebo for the prevention of antibiotic-associated diarrhea in adult patients. The x-axis depicts effect size, with black dot denoting the relative risk and the line indicating 95% CI, with the size of the grey box proportional to the study size. Relative risks to the left of RR = 1 denote study favored the probiotic, RR to the right denotes the study favors the placebo. Overall, pooled RR was 0.47 (0.35-0.63).

Pooling the results from five trials (involving 1076 subjects), a significantly protective effect of *S. boulardii* was found (pooled RR = 0.43, 95% CI: 0.23-0.78). As an alternative, some meta-analyses have done sensitivity analysis, separating out sub-groups by patient type (e.g. just adults or just children) or by type of probiotic strain[123,142]. One sensitivity analysis found with sufficient evidence that only two probiotic strains had significant efficacy for the prevention of AAD, i.e. *S. boulardii* and *L. rhamnosus* GG. However, *L. rhamnosus* GG probiotics may be contraindicated that this strain is susceptible when the patient is prescribed a type of antibiotic[123]. Although many meta-analyses have been done, pooling studies must be performed with these limitations in mind.

CDI

Epidemiology of *C. difficile* disease: For over two decades, *Clostridium difficile* infections continue to persist as a leading cause of nosocomial gastrointestinal illness[143-146]. The incidence rates of CDI have been increasing globally. In the US, CDI doubled to 301 200 cases from 2001 to 2005[147]. It is estimated that 450 000-750 000 CDI cases will occur per year by 2010 in the US[148,149]. Outbreaks of an emergent strain, BI/NAP1/027, caused large outbreaks of severe CDI with a high mortality rate in Canada between 2003-2005[150]. Studies have documented that CDI extends patients' hospital stays from 4 to 36 d[151-154]. In a study of 1034 CDI cases in Massachusetts in 2000, the average cost ranged from $10 212-$13 675/patient[155], leading to a nation-wide cost of $3.2 billion/year for CDI. There are only two standard antibiotics for CDI (vancomycin and metronidazole) and the response rate of metronidazole has been declining[152]. In addition, 20%-60% of patients may develop recurrent episodes of CDI despite additional antibiotic treatment. Although other investigational antibiotics are under development, no new antibiotics are superior to the two standard antibiotics.

Efficacy evidence for *C. difficile* disease: Probiotics may offer promise as an adjunctive therapy (given along with standard antibiotics vancomycin or metronidazole) for CDI. A meta-analysis of six randomized controlled trials of different probiotics (*S. boulardii*, *Lactobacillus rhamnosus* GG, *L. planatarum* 299v, and a mix of *L. acidophilus* and *Bifidobacterium bifidum* showed probiotics, in general, had a overall significant efficacy to prevent subsequent recurrences of *C. difficile* disease (RR = 0.59, 95% CI: 0.41-0.85, P = 0.005)[123]. However, due to limited number of trials, no meta-analysis was conducted for one probiotic strain.

S. boulardii shows promise for *C. difficile* infection control at several levels: it produces a 54-kDa serine protease that directly degrades *Clostridium difficile* toxins and also directly destroys the colonic receptor site for *C. difficile*[76], *S. boulardii* increases the immune response to *Clostridium difficile* toxins A and B[108], and animal models of *C. difficile* disease respond to this yeast. In addition, case reports or small case series of patients with recurrent *C. difficile* diarrhea treated with *S. boulardii* showed improvement. Toothaker and Elmer found that significantly fewer (51%) hamsters challenged with *C. difficile* died if given *S. boulardii* (5% solution) compared with the saline controls (80%)[156]. Subsequent studies confirmed this efficacy in other animal models: gnotobiotic mice[41,157,158], rats[89,103], and turkeys[159]. Since 1989, case reports or small case series have reported that patients with recurrent CDAD were either cured or improved by *S. boulardii* treatment[40,59, 160,161].

Table 3 Randomized controlled trials for the treatment of *C. difficile* disease using *S. boulardii*

Ref.	Treatment groups	Study population	Daily dose: cfu/d (mg/d)	Duration of treatment (wk)	Follow-up (wk)	*C. difficile* recurrence in probiotic group	*C. difficile* recurrence in placebo group
McFarland *et al*[53]	*S. boulardii vs* placebo	124 adult patients on varied doses of vancomycin or metronidazole; recurrent and initial CDAD cases; 3 referral sites, US	3×10^{10} (1000 mg)	4	4	15/57 (26.3%)[a]	30/67 (44.8%)
Surawicz *et al*[60]	*S. boulardii vs* placebo	168 adult patients recurrent CDAD; on vancomycin (2 g/d, $n = 32$) or V (500 mg/d, $n = 83$) or M (1 g/d, $n = 53$); 4 referral sites, US	2×10^{10} (1000 mg)	4	4	V (2 g/d) 3/18 (17%)[a]; V (500 mg/d) 23/45 (51%); M (1 g/d) 13/27 (48.1%)	V (2 g/d) 7/14 (50%); V (500 mg/d) 17/38 (44.7%); M (1 g/d) 13/26 (50%)

[a]$P < 0.05$, probiotic *vs* controls. V: Vancomycin; M: Metronidazole.

There was a case report[162] and two small case series with 3-4 patients[163,164] with recurrent *C. difficile* disease who seemed to have responded to *S. cerevisiae* administration. However, since no comparison groups or controls were used, there is no foundation to claim the efficacy of other strains of Saccharomyces for *C. difficile* disease.

There have been two randomized, double blinded, controlled trials for the use of *S. boulardii* and antibiotics for patients with recurrent *C. difficile* disease (Table 3)[53,60]. In a multisite, randomized, double-blinded, placebo-controlled trial of 168 patients with recurrent *C. difficile* disease, standard antibiotics were combined with *Saccharomyces boulardii* or placebo[60]. Three antibiotic regimens were used for 10 d: either high-dose vancomycin (2 g/d), low-dose vancomycin (500 mg/d) or metronidazole (1 g/d). Then either *S. boulardii* or placebo (1 g/d for 28 d) was added to the antibiotic treatment. The patients were followed up for two months for subsequent *Clostridium difficile* recurrences. A significant decrease in recurrences was observed only in patients treated with the high-dose vancomycin and *S. boulardii* treatment (16.7%) compared with patients who received high-dose vancomycin and placebo (50%, $P = 0.05$). There were no significant reductions in recurrence rates in either the low-dose vancomycin or metronidazole treatment group, regardless if *S. boulardii* was used. No serious adverse reactions were noted in any of these patients. By the end of the antibiotic treatment, only high-dose vancomycin completely cleared *C. difficile* toxin from the colon, low-dose vancomycin and metronidazole could not clear the toxin. A subsequent investigation of 163 patients with recurrent CDI confirmed this finding[165]. In this study, stool samples were assayed at the end of antibiotic treatment to determine if there was a difference in toxin clearance by the type or dose of antibiotic. Treatment with high-dose (2 g/d) of vancomycin completely cleared *C. difficile* toxin, but 11% of those treated with lower doses of vancomycin and 41% treated with metronidazole were positive for *C. difficile* at the end of antibiotic treatment. *S. boulardii* may be more effective if complete *C. difficile* toxin clearance is achieved before sole reliance upon the yeast probiotic is required.

An earlier randomized, controlled double-blinded study had also found efficacy of the combination treatment using a standard antibiotic (vancomycin or metronidazole) and *S. boulardii* for patients with *C. difficile* disease[53]. This study did not control the dose or duration of either vancomycin or metronidazole (it was under the physician's discretion), but randomized patients to either *S. boulardii* (1 g/d) or placebo for 28 d. All patients were followed up for two months for subsequent recurrences. Approximately half of the enrolled patients had their first episode and half had recurrent *C. difficile* disease. Twenty-six percent of the 57 patients given *S. boulardii* had a recurrence of *C. difficile* disease, which was significantly fewer as compared with 45% of the 67 given placebo ($P < 0.05$). The strongest effect was found in the 60 patients with recurrent *C. difficile* disease; significantly fewer patients (35%) given *S. boulardii* had a recurrence compared with 65% of patients given placebo ($P = 0.04$). Two adverse reactions were associated with *S. boulardii* (thirst and constipation). No randomized controlled trials using stains of *S. cerevisiae* for this disease were found in the literature.

H. pylori

Epidemiology of *H. pylori*: *H. pylori* colonizes the gastric mucosa and usually induces a chronic, asymptomatic carrier state, but some people may develop gastroduodenal ulcers as a result. Carriage of *H. pylori* is a risk factor for developing gastric lymphoma or adenocarcinoma in later life. The prevalence of *H. pylori* carriage is 50%-80% worldwide[166,167]. Standard treatment for *H. pylori* involves a two-week course of triple therapy (usually clarithromycin, amoxicillin and an acid suppressor such as lansoprazole or omeprazole), that also has gastrointestinal side effects.

Efficacy for *H. pylori*: *S. boulardii* induces morphologic changes in *H. pylori* cells consistant with cellular damage[21] and was shown to reduce 12% of *H. pylori* colonization in infected children in one trial[46]. Of four randomized controlled trials testing *S. boulardii* in *H. pylori* infections, two were in children[46,168] and two (Table 4) were in adults[42,134]. Cremonini *et al*[134] first explored the efficacy of probiotics to eradicate *H. pylori* in 85 asymptomatic carriers. They

Table 4 Randomized, controlled trials for acute disease conditions using *S. boulardii*

Ref.	Treatment groups	Study population	Daily dose: cfu/d (mg/d)	Duration of treatment	Follow-up (wk)	Outcome in probiotic	Outcome in controls
	Helicobacter pylori						
Cremonini *et al*[134]	*S. boulardii* (Codex, SmithKine Beecham, Italy) *vs* placebo *vs* LGG *vs* mix (*L acid* and *Bifid lactis*)	85 asymptomatic carriers *H. pylori* 22 SB and 21 placebo; all received triple therapy	5×10^9 (nr)	2 wk	2	Eradicated in: 17/21 (81%) same for LGG and mix Any symptoms: 4/21 (19%)[a]	Placebo, 16/20 (80%) Any symptoms: 12/20 (60%)
Cindoruk *et al*[42]	*S. boulardii* (Reflor, Turkey) *vs* placebo	124 adults with *H. pylori* dyspepsia, all received triple therapy	2×10^{10} (1000 mg)	2 wk	6	Eradication 44/62 (71%) Epigastric distress: 9 (14.5%)[a]	Eradication 37/62 (59.7%) Distress: 27 (43.5%)
	Acute adult diarrheas						
Hochter *et al*[169]	*S. boulardii* *vs* placebo	92 German outpatient adults	8×10^9 (300-600 mg)	8 d	0	Diarrhea score reduction: -17.2 + 6.8[a]	Score: -13.6 + 8.7
Mansour-ghanaei *et al*[51]	*S. boulardii* (UltraLevure, France) *vs* placebo. Both groups got metro and lodoquinol	57 enrolled, 54 done; adults with *E. histolytica* amoebic dysentery	1.5×10^{10} (750 mg)	10 d	4	Cured: 27/27 (100%)[a]	Cured: 5/27 (19%)
	Enteral feeding-related diarrhea						
Tempe *et al*[61]	*S. boulardii* *vs* placebo	40 adults in ICU	1×10^{10} (nr)	11-21 d	0	34/389 days of diarrhea (8.7%)[a]	63/373 days of diarrhea (16.9%)
Schlotterer *et al*[171]	*S. boulardii* *vs* placebo	20 enrolled, 18 done, burnt adults 18-70 yr	4×10^{10} (2000 mg)	8-28 d	0	3/204 days of diarrhea (1.5%)[a]	19/208 days of diarrhea (9.1%)
Bleichner *et al*[39]	*S. boulardii* *vs* placebo	131 enrolled, 128 done, adults in ICU	4×10^{10} (2000 mg)	3 wk	0	50/648 days of diarrhea (7.7%)[a]	87/683 days of diarrhea (12.7%)
	Traveler's diarrhea						
Kollaritsch *et al*[175]	*S. boulardii* *vs* placebo	832 Austrian tourists to hot climates	2×10^9 (250 mg)	5 d before trip and mean 21 d trip	0	143/426 (34%)[a]	173/406 (43%)
Kollaritsch *et al*[175]	*S. boulardii* *vs* placebo	805 Austrian tourists to hot climates	5×10^9 (500 mg)	5 d before trip and mean 21 d trip	0	127/399 (32%)[a]	173/406 (43%)
Kollaritsch *et al*[49]	*S. boulardii* *vs* placebo	713 Austrian tourists to hot climates	2×10^9 (250 mg)	5 d before trip and mean 21 d trip	0	121/352 (34%)[a]	141/361 (39%)
Kollaritsch *et al*[49]	*S. boulardii* *vs* placebo	664 Austrian tourists to hot climates	2×10^{10} (1000 mg)	5 d before trip and mean 21 d trip	0	87/303 (29%)[a]	141/361 (39%)
Bruns *et al*[176]	*S. cerevisiae* Hansen (CBS 5926) *vs* active treatment[1]	60 tourists in Tunisia with traveler's diarrhea, 43 done	1.0×10^{10} (600 mg)	5 d	0	Duration diarrhea 2.1 d	1.4 days of diarrhea[a]

[a]$P < 0.05$, probiotic *vs* controls; [1]Strain currently known as *S. boulardii*.

randomized patients to one of three probiotic treatments (*S. boulardii*, *L. rhamnosus* GG or a mixture of *L. acidophius* and *Bifidobacterium lactis*) or placebo for 14 d. All patients were treated with the triple therapy for the first week. By the end of the second week, eradication rates were similar for all groups (81% for *S. boulardii vs* 80% of placebo). A promising result of this study was a lower rate of antibiotic-associated diarrhea (5%) in all the probiotic groups compared with 30% of the placebo group. A subsequent study by Cindoruk *et al*[42] assessed *S. boulardii* for both eradication of *H. pylori* and the reduction of side-effects of the standard triple treatment. This study enrolled 124 adults with *H. pylori* dyspepsia in Turkey who were receiving the triple therapy and randomized to either 1 g of *S. boulardii* (2×10^{10}/d for 2 wk) or placebo. Patients were followed up for six weeks for side-effects and *H. pylori* clearance. Although there was no significant difference in *H. pylori* eradication (71% in *S. boulardii vs* 60% in placebo), significantly fewer patients randomized to *S. boulardii* reported epigastric distress (14.5%, $P < 0.05$) compared with placebo (43.5%), as well as lower global dyspepsia symptom scores. The frequency of AAD was also significantly reduced in those receiving *S. boulardii* (14.5%) compared with the placebo group (30.6%). These studies indicate that *S. boulardii* may not be effective in eradicating *H. pylori* itself, but it is effective in reducing the side-effects of the standard triple therapy.

Acute adult diarrheas

Epidemiology of acute adult diarrhea: Acute adult diarrhea is a broad classification for diarrhea that includes illnesses that may develop quickly, but are typically short-lived and are sporadic. The etiologies of acute adult diarrhea may include infectious agents (*Entamoeba histolytica*, *E. coli*, or Salmonella) or may be idiopathic. Diarrhea in adults occurring in outbreaks or due to other situations (antibiotic-associated diarrhea, *C. difficile*-associated diarrhea, traveler's diarrhea, inflammatory bowel disease or irritable bowel disease) are not included in this classification.

Clinical efficacy for acute adult diarrhea: Two randomized controlled trials using *S. boulardii* showed that this probiotic may be effective in treating acute diarrhea due to a variety of causes. Hochter *et al*[169] enrolled 92 German

adult outpatients with acute diarrhea and randomized them to *S. boulardii* (8×10^9/d) or placebo for eight days. By the third day, the 43 given *S. boulardii* had a significantly lower diarrhea severity score (5.5 ± 6.8, $P = 0.04$) compared with the 49 given placebo (6.7 ± 8.7). Mansour-Ghanaei *et al*[51] enrolled 57 patients with acute *Entamoeba histolytic* dysentery and treated them with *S. boulardii* (1.5×10^{10}/d for 10 d) or nothing (control). Both groups also received metronidazole and iodoquinol for 10 d. At the end of four weeks, all the patients given *S. boulardii* were cured compared with 19% of those not given the yeast. Unfortunately, the number of trials in this area is small and the etiologies were different in the two trials, so conclusions are limited. No randomized controlled trials using stains of *S. cerevisiae* for this disease were found in the literature.

Enteral nutrition-related diarrhea

Epidemiology of enteral nutrition-related diarrhea: Diarrhea is a common complication associated with enteral tube feeding and may result in a loss of nutrition in an already seriously ill patient. The frequency of diarrhea in enteral tube fed patients has been reported as high as 50%-60%[170] and complications may include life-threatening acidosis, increased morbidity and mortality and increased healthcare costs. Schneider *et al*[102] reported a significant increase in short-chain fatty acids in 10 enteral-fed patients receiving *S. boulardii* (1 g/d for 6 d) compared with 15 healthy controls.

Clinical efficacy for enteral nutrition related diarrhea: Three randomized, controlled trials have assessed the ability of *S. boulardii* to reduce diarrhea in patients receiving this type of nutritional intake (Table 4). Tempe *et al*[61] compared 40 enteral-fed patients randomized to either *S. boulardii* (1×10^{10}/d) or placebo for 11-21 d and found significantly fewer patients (8.7%) given *S. boulardii* developed diarrhea compared with placebo (16.9%). Schlotterer *et al*[171] randomized 18 patients with burns who were receiving enteral nutrition to *S. boulardii* (4×10^{10}/d) or placebo for 8-28 d. Those given *S. boulardii* suffered fewer diarrhea days (3/204 d, 1.5%) than those given placebo (19/208 d, 9.1%, $P < 0.001$). The efficacy of *S. boulardii* was confirmed in a later study of 128 intensive care unit (ICU) patients randomized to either *S. boulardii* (4×10^{10}/d) or placebo for 21 d[39]. Those given *S. boulardii* reported significantly fewer diarrheal days (7.7%) than those given placebo (12.7%). No adverse reactions associated with *S. boulardii* were reported in any of these three trials.

Traveler's diarrhea

Epidemiology of traveler's diarrhea: Traveler's diarrhea (TD) is a common health complaint among travelers; globally 12 million cases are reported every year[172]. Rates of TD range from 5% to 50%, depending upon the destination, with equatorial countries having the highest rates of TD. TD usually occurs as sporadic cases, but outbreaks of TD may occur in large groups traveling or located together (tourists on cruise ships, group tours, military personnel, disaster-relief groups). The etiologies of TD vary widely, but commonly include enterotoxigenic and entroaggregative *E. coli*, *Campylobacter jejuni*, *Salmonella typhimurum*, viruses (Norwalk or Rotavirus) or parasites (*Entamoeba histolytica*, *Giardia lamblia*). Traditional preventive measures including frequent handwashing, avoiding high-risk foods and water and ingestion of bismuth subsalicyclate showed only modest protection[173].

Clinical efficacy for TD: A meta-analysis of 12 randomized, controlled trials of various probiotics (including *S. boulardii*, *Lactobacilli* and mixtures of different probiotic strains) for the prevention of TD found a significant reduction in the risk of TD if probiotics were used (RR = 0.85, 95% confidence interval 0.79-0.91)[174]. Two randomized controlled trials have been done with *S. boulardii* that included a total of four different probiotic treatment arms (Table 4). Kollaritsch *et al*[175] enrolled 1231 Austrian tourists traveling to hot climates and randomized travelers to either of two doses of *S. boulardii* (250 or 500 mg/d) or placebo for 3 wk. The treatment was started 5 d prior to the trip and continued through the duration of the trip. Traveler's diarrhea developed in 43% given placebo and significantly fewer in those given the low-dose *S. boulardii* (34%) and the higher dose *S. boulardii* (32%). A second study by Kollaritsch *et al*[49] was done with 3000 Austrian tourists traveling to northern African, the Middle East and the Far East. Travelers were randomized to either 250 mg/d (5×10^9) *S. boulardii*, 1000 mg/d (2×10^{10}) *S. boulardii* or placebo, starting five days before leaving and throughout the duration of their trip (median of 3 wk). Only 1016 (34%) completed the study, but *S. boulardii* significantly reduced the incidence of traveler's diarrhea in a dose-dependent manner. Of the travelers given placebo, 39% developed diarrhea whereas only 34% of those given the lower dose and 29% of those given the higher dose of *S. boulardii* developed traveler's diarrhea ($P < 0.05$). No adverse reaction was noted by the travelers in either of these studies.

Bruns *et al*[176] tested *S. cerevisiae* Hansen CBS 5926 (Perenterol®, Germany) to treat travelers who had developed TD in Tunisia. Travelers with TD were randomized to 600 mg of *S. cerevisiae* (1×10^{10}/d) for five days or an active control treatment and the duration of their TD tracked. Of 60 enrolled, 43 completed the trial, but the duration of diarrhea was not significantly different between those given *S. cerevisiae* (2.1 d) and those given ethacridine-lactate and tannalbuminate (1.4 d). This strain is known as *S. boulardii* outside of Germany. No other randomized controlled trials have been done for the treatment of TD using *S. boulardii*. These limited numbers of studies indicate that probiotics may be more effective in preventing TD, rather than treating the diarrhea once it becomes symptomatic.

CLINICAL EFFICACY: CHRONIC DISEASES

S. boulardii has been tested for clinical efficacy in several types of chronic diseases including Crohn's disease, irri-

table bowel syndrome, giardiasis and human immunodeficiency virus (HIV)-related diarrhea.

Inflammatory bowel disease

Epidemiology of inflammatory bowel disease: inflammatory bowel diseases (IBDs) are chronic, immune-related inflammatory diarrheas, which include ulcerative colitis, pouchitis and Crohn's disease. The incidence of Crohn's disease varies by countries, with the highest incidences (8-66/100 000) found in Wales, New Zealand, Canada, Scotland, France, Netherlands, intermediate rates (4-7/100 000) in UK, USA, Europe, Australia, and the lowest rates (0.2-3/100 000) in South America, China, Korea, Japan[177]. In Crohn's disease, typically the lower small intestine and colon are the most affected organs. Symptoms include diarrhea, abdominal pain or cramping and loss of appetite. Unlike ulcerative colitis, lesions of Crohn's disease are deep and patchy and often involve thickened areas resulting in intestinal obstruction, which can be life threatening. Disruption of the intestinal wall also allows intestinal bacteria to translocate and incite the immune system. Complications of Crohn's disease are common, in that 40% of Crohn's patients report lower GI bleeding, intestinal obstruction, or perforation[178]. Lifetime risk for surgery in Crohn's patients is extremely high (70%-80%)[179]. Unfortunately, the cause of Crohn's disease is not known, so current therapies are directed at symptom relief. But 10%-60% suffer recurrences of Crohn's disease after treatment is completed[180].

Clinical efficacy for IBD: Guslandi *et al*[48] enrolled 25 adults with mild to moderate ulcerative colitis in a pilot study and treated them with a combination of mesalazine (3 g/d) and *S. boulardii* (1.5×10^{10}/d) for 4 wk. All of these patients had histories of poorly tolerated steroid courses. Most (68%) of the patients responded to the probiotic treatment. This study had a promising result, but the implications were uncertain as patients treated for only a short time, were not followed up for subsequent flare-ups of their disease and there was no control group in this pilot study. Garcia Vilelea *et al*[62] enrolled 31 patients with Crohn's disease in remission (at the time of enrollment). All patients continued their maintenance medications during the trial (mesalamine, azathioprine or others). Patients were randomized to either *S. boulardii* (2×10^{9}/d) for three months or placebo. Those treated with *S. boulardii* were found to have a significant reduction in colonic permeability compared with those given placebo, thus reducing the risk of translocation in these patients.

Two randomized controlled clinical trials have been done testing *S. boulardii* for patients with Crohn's disease (Table 5)[47,57]. Plein *et al*[57] randomized 20 patients with Crohn's disease to either 750 mg of *S. boulardii* (1.5×10^{10}/d) or placebo for seven weeks. All patients continued their maintenance medications during the trial. At the end of seven weeks, patients treated with *S. boulardii* were significantly improved (mean = 3.3 ± 1.2 stools/d, $P < 0.05$) compared with the placebo group (mean = 4.6 ± 1.9 stools/d). Guslandi *et al*[47] studied the effect of *S. boulardii* on the rate of Crohn's disease relapses in a study of 32 patients with Crohn's disease in Italy. Adults (23-49 years old) who were in remission were randomized to either 1 g of *S. boulardii* (2×10^{10}/d) and mesalamine (2 g/d) or mesalamine alone (3 g/d) for 6 mo. Significantly fewer patients treated with *S. boulardii* (6%, $P = 0.04$) relapsed than the control group (38%). No adverse reactions associated with *S. boulardii* were reported in any of these trials.

Crohn's disease is a challenging condition for clinical trials in that it is sporadic, has no defined etiology, has multiple measurable disease outcome and requires lengthy treatment and follow-up period. *S. boulardii* offers promise to this group of patients who are in need of a safe and effective therapy for this chronic condition. The probiotic protection seems to be strain specific. A study tested another strain of *S. cerevisiae* (Baker's yeast) in 19 adults with Crohn's disease, but found the disease was worse in those receiving this strain than in controls[181].

Irritable bowel syndrome

Epidemiology of irritable bowel syndrome: Irritable bowel syndrome (IBS) is a frequent disorder characterized by a triad of symptoms (bloating, abdominal pain, and intestinal transit disturbances). The prevalence of IBS is high (3%-20%) in the general population and IBS may account for 25%-50% of gastroenterologist practice[182]. Consequences of IBS include worse quality of life[183], higher health care costs ($1.7 billion in health care costs)[184], and higher incidences of depression[185].

Clinical efficacy for IBS: Standard treatments target symptom relief, but have not been more effective than placebo. IBS is a recent focus of probiotic clinical trials. A meta-analysis of 20 randomized, controlled trials (with 23 treatment arms) including 1404 subjects found a pooled relative risk (RR) for improvement in global IBS symptoms in 14 probiotic treatment arms (RR = 0.77, 95% CI: 0.62-0.94)[36]. One of those trials (Table 5) randomized 34 patients with IBS to *S. boulardii* (9×10^{9}/d) or placebo for four weeks[52]. A significant decrease in the daily number of stools was found in the *S. boulardii* group and 87.5% of this group reported their IBS had improved compared with 72% of the placebo group ($P < 0.05$). No adverse reactions were noted in this trial. More trials using *S. boulardii* for IBS are required.

Giardiasis

Epidemiology of giardiasis: This condition is characterized by long lasting diarrhea with symptoms ranging from mild to severe diarrhea, weight loss, abdominal pain and weakness. The incidence may be high in developing countries such as Colombia (13%) and lower in developed countries such as Germany (12/100 000)[186,187]. It is also found in people enjoying outdoor hiking and camping who drink contaminated untreated water that appears clean.

Clinical efficacy for giardiasis: Besirbellioglu *et al*[38] randomized 65 adults with giardiasis in Turkey to either *S. boulardii* (1×10^{10}/d) or placebo for 10 d. Both groups

Table 5 Randomized, controlled trials for chronic disease conditions using *S. boulardii*

Ref.	Treatment groups	Study population	Daily dose: cfu/d (mg/d)	Duration of treatment and follow-up	Outcome in probiotic	Outcome in controls
	Crohn's disease					
Plein *et al*[57]	*S. boulardii* (Perenterol®) *vs* placebo	20 enrolled with Crohn's, 17 done, all on maintenance medications	1.5×10^{10} (750 mg)	7 wk	3.3 ± 1.2 stools/d at week 9[a]	4.6 ± 1.9 stools/d at week 9
Guslandi *et al*[47]	*S. boulardii* (Perenterol®) + mesalamine (2 g/d) *vs* mesalamine alone (3 g/d)	32 with Crohn's in remission in Italy (23-49 yr old)	2×10^{10} (1000 mg)	6 mo	1/16 (6%) relapse[a]	6/16 (38%) relapse
	Irritable bowel syndrome					
Maupas *et al*[52]	*S. boulardii vs* placebo	34 with IBS	9×10^{9} (nr)	4 wk	Mean decrease in #stools/d: -2.2/d[a] Improved: 14/16 (87.5%)[a]	Mean decrease -0.5/d Improved: 13/18 (72%)
	Giardiasis					
Besirbellioglu *et al*[38]	*S. boulardii* + metro (2250 mg/d) *vs* placebo + metro	65 adults with giardiasis	1×10^{10} (500 mg)	10 d with 4 wk f/up	100% cured, 0% giardia cysts[a]	100% cured 6/35 (17%) cysts present
	HIV-related diarrhea					
Saint- Marc *et al*[189]	*S. boulardii vs* placebo	35 French AIDS patients with chronic diarrhea	6×10^{10} (3000 mg)	7 d	11/18 (61%) cured[a]	2/17 (12%)

[a]$P < 0.05$, probiotic *vs* controls. IBS: Irritable bowel syndrome; HIV: Human immunodeficiency virus; AIDS: Acquired immune deficiency syndrome; f/up: Follow-up time.

also received metronidazole for the same duration. Two weeks later, both groups reported a resolution of their diarrhea, but none of those on *S. boulardii* had detectable giardia cysts, while significantly more (17%) on placebo still carried giardia cysts.

HIV diarrhea

Epidemiology of HIV-related diarrhea: Patients infected with HIV are susceptible to a variety of diseases and may also develop chronic life-threatening diarrhea. The prevalence of diarrhea in HIV patients ranges from 50%-60% in developed countries and nearly 100% in developing countries[188].

Clinical efficacy for HIV-related diarrhea: A blinded, placebo-controlled dose-ranging study was done in 11 HIV positive patients who had chronic diarrhea[45]. *S. boulardii* was given at 1-3 g/d and six patients reported diarrhea was controlled while taking 3 g/d after one month, while lower doses of *S. boulardii* were not as effective. Patients with HIV-associated diarrhea seem to be one group that requires a higher than typical dose of *S. boulardii*. Saint-Marc *et al*[189] randomized 35 Stage IV AIDS adult patients with chronic diarrhea to either *S. boulardii* (3 g/d or 6×10^{10}/d) or placebo for one week (Table 5). Significantly more of those given *S. boulardii* (61%, $P = 0.002$) had their diarrhea resolved compared with placebo (12%) patients. *S. boulardii* was well tolerated by all HIV patients even treated with high doses of 3 g/d in these two studies.

SAFETY OF *S. BOULARDII*

The use of a living organism as therapy increases the potential risk in four general areas: transfer of antibiotic-resistance genes, translocation of the living organism from the intestine to other areas of the body, persistence in the intestines and the development of adverse reactions. The first three theorical concerns are of minimal impact on *S. boulardii*. Unlike other bacterial strains of probiotics, such as *Enterococcus faecium* and *Lactobacillus rhamnosus*, which have been shown to acquire antibiotic-resistance genes, *S. boulardii* has not developed any antibiotic or antifungal resistance[190,191]. Data from animal models shows reduced translocation with *S. boulardii* treatment[64,89], unlike other strains of *S. cerevisiae*[192]. As pharmacokinetic studies indicate, *S. boulardii* does not persist 3-5 d after oral ingestion is discontinued, so persistence is not a concern for this probiotic[118].

S. boulardii has been used as a probiotic since the 1950s in Europe and has been investigated in clinical trials worldwide. Safety and adverse event data collected during clinical trials, when patients are closely monitored for problems associated with the investigational treatment, has documented a remarkable safety profile of *S. boulardii* in adults. A wide diversity of adult patients have been followed up who were either randomized to *S. boulardii* as part of a clinical trial or were documented in case series or case reports for various disease indications (TD, $n = 1596$; AAD, $n = 958$; adult acute diarrhea, 156; enteral tube feeding, $n = 103$; IBD, $n = 66$; IBS, $n = 16$, HIV-related diarrhea, $n = 18$ and giardia infections, $n = 50$). These studies provide safety data for a total of 2963 adult patients and the only adverse reactions associated with *S. boulardii* was thirst (in 5 patients) and constipation (in 8 patients) in a trial of patients with *C. difficile* infections[53]. No case of *S. boulardii* fungemia has been reported in these diverse patient populations enrolled in clinical trials.

Infrequent cases of fungemia have been reported in case reports or case series in the literature. Most of the adult cases of *S. boulardii* fungemia are with serious co-

Table 6 Summary of recommendations for clinical use of *S. boulardii* in adults

Use for disease	Dose (mg/d)	Duration	Adjunct to	Strength of evidence[1]
Prevention of antibiotic associated diarrhea	500-1000	During antibiotics with additional 3 d to 2 wk after	Nothing	++++
Prevention of Traveler's diarrhea	250-1000	Duration of trip (3 wk)	Nothing	+++
Enteral nutrition-related diarrhea	2000	8-28 d	Nothing	++
H. pylori symptoms	1000	2 wk	Standard triple therapy	++
Treatment of *Clostridium difficile* infections	1000	4 wk	Vancomycin or metronidazole	+
Acute adult diarrhea	500-750	8-10 d	Nothing	+
Inflammatory bowel disease	750-1000	7 wk to 6 mo	Mesalamine	+
Irritable bowel syndrome	500	4 wk	Nothing	+
Giardiasis	500	4 wk	Metronidazole	+
HIV-related diarrhea	3000	7 d	Nothing	+

[1]Strength of evidence, + (weak, needs more randomized controlled trials) to ++++ (strong, efficacy and safety are evidence based from numerous large randomized controlled trials).

morbidities and have central venous catheters. Most cases responded well to treatment with fluconazole or amphotericin B[193]. Some cases developed fungemia, not from the direct ingestion of *S. boulardii* probiotics, but acquired the yeast from contaminated environmental fomites[194]. Fungemia from *S. cerevisiae* (non-boulardii strains) have also been reported and are similar to *S. boulardii* cases, but have poorer prognosis[195,196]. A challenge to determining valid incidence of fungemia is the lack of available advanced yeast identification assays which can distinguish *S. cerevisiae* from *S. boulardii*.

CONTRAINDICATIONS/PRECAUTIONS

Even though fungemia with *S. boulardii* is infrequent, it may be prudent to closely follow up the adult inpatients who are severely ill or in intensive care units and have central catheter for episodes of unexplained fever. Some studies have recommended not to give *S. boulardii* to immunosuppressed patients or those with central catheters to reduce this risk[108].

Unlike many bacterial probiotics, there are few drug or antibiotic interactions with *S. boulardii*. However, if patients are on anti-fungal medications, a staggered dose regime (at least 4 h apart) is suggested[45].

CONCLUSION

The use of *S. boulardii* as a therapeutic probiotic is supported by its mechanisms of action, pharmacokinetics, and efficacy from animal models and clinical trials. The overall safety profile for *S. boulardii* is beneficial. *S. boulardii* can be recommended for several diseases. Other strains of Saccharomyces may have probiotic properties, but clinical efficacy evidence for other strains are still deficient. Guidelines for the most effective use of *S. boulardii* are given in Table 6 and generally, the daily dose is typically $> 10^9$/d, the duration of treatment can vary from 7 d to 6 mo and it may be given alone or as an adjunctive treatment, depending upon the disease indication. *S. boulardii* is significantly effective for the prevention of AAD and prevention of TD. Trials also show evidence for *S. boulardii* in the reduction of side-effects of *H. pylori* treatment and the prevention of enteral nutrition-related diarrhea. More clinical trials are encouraged for the treatment of chronic diseases (Crohn's disease, irritable bowel syndrome and HIV-related diarrhea) and the prevention of *C. difficile* disease recurrences.

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TOPIC HIGHLIGHT

Metin Basaranoglu, PhD, Associate Professor, *Series Editor*

Understanding mechanisms of the pathogenesis of nonalcoholic fatty liver disease

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Abstract

A central issue in the understanding of the pathogenesis of nonalcoholic fatty liver disease is the problem of the underlying mechanisms which are not fully understood. In the setting of excessive central adiposity, insulin resistance is the major underlying cause of fat accumulation in hepatocytes. Because of the difficulties with human trials, several animal models have been developed for this purpose mainly characterized as follows: genetically disturbed or murine fatty liver, methionine-choline deficient diet fed or murine steatohepatitis, and high-fat or sucrose diet fed models. Although these animal models have provided useful information, none of them accurately reflect genetic, metabolic and biochemical characteristics of the human disease.

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Key words: Nonalcoholic fatty liver disease; Pathogenesis; Rat; Rodents

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OBESITY AND NONALCOHOLIC FATTY LIVER DISEASE

Nonalcoholic fatty liver disease (NAFLD) is one of the most prevalent forms of chronic liver disease[1-4]. The reported prevalence of NAFLD in developed countries is 30% and 13% in adults and children, respectively. It is likely that in type 2 diabetes mellitus, NAFLD is one of the consequences of obesity. The prevalence of NAFLD in the obese population is nearly 95%. Factors contributing to NAFLD include sedentary life style, and increased consumption of foods with high-fat and high fructose corn syrup content. A cafeteria style diet which includes both high fat and fructose corn syrup is the

leading cause of obesity in the population. Furthermore, in the setting of excessive central adiposity, insulin resistance is the major underlying cause of fat accumulation in the liver[5-11]. NAFLD is characterized by hepatic fat accumulation in hepatocytes (> 5% of liver weight).

NONALCOHOLIC STEATOHEPATITIS

Nonalcoholic steatohepatitis (NASH), as a subgroup of NAFLD, is characterized by chronic, progressive liver pathology which has the ability to lead to advanced fibrosis, cirrhosis, hepatocellular carcinoma, and liver-related death[1,2,4,5]. The prevalence of NASH is 3% among adults. The mechanisms underlying NASH pathogenesis are not fully understood. One of the hypotheses has been termed the "two-hit" theory[5,6]. According to this paradigm, NAFLD is a result of inappropriate fat storage or ectopic fat accumulation and the primary abnormality is most likely insulin resistance which leads to the accumulation of triglycerides (TGs) within the hepatocytes. After the first hit of steatosis, the second hit of oxidative stress leads to hepatocyte injury and inflammation.

Animal models

There are several types of animal model used for NAFLD studies, and these are mainly characterized as follows: genetically disturbed or murine fatty liver, methionine-choline deficient (MCD) diet fed mice or murine steatohepatitis model and feeding high-fat and/or sucrose diets with or without high caloric intake model[12-24]. Manipulation of the mouse genome and production of new animal models, such as leptin-deficient ob/ob mice, leptin-resistant db/db rats or knockout mice regarding a special character, have given us great opportunities for research[12-16]. However, it is not possible to extrapolate all the information gained from these animal models into knowledge on the human species as there are some fundamental differences regarding their genetics, mediators, and the mechanisms of the events. For example, although obese patients have higher circulating leptin levels, ob/ob mice exhibit complete leptin deficiency. Additionally, leptin has some immunologic functions, besides its metabolic regulatory capacity. Secondly, although insulin resistance is a universal feature of patients with NASH, the MCD model is not insulin-resistant and not obese[15]. MCD mice have increased insulin hypersensitivity, and their serum has both insulin and glucose levels lower than mice fed a standard diet.

High caloric intake models

With regard to the problems mentioned above, it is reasonable to try to find a suitable model for investigating the mechanism behind human NASH. Actually, there have been several attempts to mimic human species by feeding mice with high fat and/or sucrose diets with or without high caloric intake in order to produce obesity, insulin resistance, and NASH[16-23]. The type and amount of fat, and total daily caloric intake on these diets are very important and there has been no standardized diet reported by study groups. In this regard, Lieber *et al*[25] fed Sprague-Dawley rats with a high-fat, liquid diet (71% of energy from fat which included corn, olive, and safflower oil) for 3 wk and developed a steatohepatitis model in murines which resembled human NASH. These mice displayed obesity and insulin resistance together with increased hepatic tumor necrosis factor (TNF)-α, TNF-α messenger RNA, cytochrome 2E1, cytochrome 2E1 mRNA, increased oxidative stress and lipid peroxidation, fatty liver histopathology, mononuclear cell infiltration, abnormal mitochondria and increased collagen in the liver. Bruce *et al*[26] used a high fat diet (45% kcal from fat, 20% kcal protein, 35% kcal carbohydrate) and Tetri *et al*[27] fed mice with 45% calories in the chow from fat and 30% of the fat in the form of partially hydrogenated vegetable oil (28% saturated, 57% monounsaturated fatty acids, 13% polyunsaturated fatty acids). Both research groups developed mice displaying obesity, insulin resistance and NASH.

There is substantial diversity within and between mouse strains resembling phenotypic variations in human populations. C57 BL6J mice have usually been chosen by NASH researchers because of their predisposition to develop insulin resistance by means of diet and the availability of genetically manipulated mice[14,27-29]. These features of the strain are explained by a strong influence of the genetic background on the susceptibility to diet-induced obesity and insulin resistance[14].

WHAT WE KNOW ABOUT THE MECHANISMS UNDERLYING NASH PATHOGENESIS?

For NASH pathogenesis, it is a prerequisite firstly to develop fatty liver which is then unusually vulnerable to various second hits or injury[5,6]. Although insulin resistance is a universal finding for both simple steatosis and NASH, only a small group of insulin-resistant patients develop NASH, even in patients with metabolic syndrome (MS). MS is the most severe form of insulin resistance and might instigate more advanced NASH. It is believed that oxidative stress and lipid peroxidation might play a central role in the transition of simple steatosis to NASH[30].

Increased production of ROS and lipid peroxidation of hepatocyte membranes and organelles promote necroinflammation, satellite cell activation and fibrosis in the liver. In this context, elevated free fatty acids (FFAs) in both circulation and the liver, mitochondrial abnormalities (dysfunction and structural abnormalities such as mega mitochondria with true crystalline inclusions), gut-derived endotoxins, ethanol secondary to gut and liver interaction, and disturbed production of adipokines should be major concerns in the development of steatohepatitis in an animal model.

Insulin resistance and peripheral lipolysis cause an

increased FFA pool in the circulation[7,21,30]. This pool is one of the major sources of hepatic TGs. FFAs are also the major source of hepatic mitochondrial, peroxisomal and microsomal ROS production. It has been reported that increased hepatic and serum FFA concentrations promote hepatic and systemic insulin resistance by the activation of PKCθ and serine phosphorylation of insulin receptor substrates. Feldstein *et al*[30] reported that FFAs promote lysosomal permeabilization, release cathepsin B which is a lysosomal protease within hepatocytes, and cause hepatocyte apoptosis and injury. In addition to these aspects, increased serum concentrations of FFAs due to obesity were found to be correlated with the severity of fibrosis in patients with NASH[31]. FFAs play a major role in the transition from simple steatosis to NASH.

High-fat diet-induced obesity and insulin resistance have been reported in Wistar rats[32,33], Sprague-Dawley rats[34,35], F344 rats[36], and in Long-Evans rats[37]. Additionally, prolonged feeding periods with high fat (59% fat), such as a 3 mo duration, promoted a greater degree of insulin resistance in Wistar rats[33]. Borst and Conover induced obesity and developed an insulin-resistant animal model by feeding them for 39 d with a high-fat (50%) inclusive diet[38]. In this model, daily caloric intake was not increased, and it was even slightly less than for the normal rat chow (12.4% fat). These mice developed increased visceral and subcutaneous fat mass, insulin resistance, elevated fasting serum insulin, decreased insulin-stimulated glucose transport in skeletal muscle, increased TNF-α expression on visceral adipose tissue, and an undetectable serum TNF-α concentration. Most importantly, serum FFA concentration was not increased significantly in both mice fed high-fat diet and controls, while liver TNF-α expression was not affected. This observation is interesting, as obesity is strongly associated with increased concentration of FFAs in the circulation.

It was previously reported that a five-fold increase of plasma FFAs caused up to 100-fold increase in plasma insulin concentrations[26,30]. Thus, FFAs are more important than TNF-α for inducing insulin resistance. TNF-α expressed in visceral adipose tissue macrophages, and maybe TNF-α in muscle, appears the major cause of systemic insulin resistance in these animal models, since FFA levels were not increased. Oxidative stress is one of the most popular proposed mechanisms of hepatocellular injury in NASH in both animal experiments and human trials. It has been reported that obesity correlates with systemic oxidative stress. Obese adults with MS have a higher plasma concentration of oxidative stress biomarkers than obese adults without MS[2,6,7]. Increased ROS production has been selectively shown in adipose tissue of obese mice. There is also some indirect evidence as to the benefit of antioxidants such as vitamin E, S-adenosylmethionine and betaine, phlebotomy to remove iron, and N-acetylcysteine for treating NAFLD. However, a causal relationship or a pathogenic link between NASH and oxidative stress has so far not been established.

Hepatocyte mitochondria are the main site of β-oxidation of FFAs and adenosine triphosphate (ATP) production is one of the crucial issues in the understanding of NASH pathogenesis. It was previously reported that mitochondrial structural abnormalities, depletion of mitochondrial DNA and ATP, and mitochondrial dysfunction are characteristics of NASH patients as well as of diet-induced and animal models[39-41]. Increased oxidative stress and lipid peroxidation products are integral components of the pathway progressing to NASH from fatty liver. Specifically, rats fed a high-fat diet have been shown to have reduced electron transport chain capacity and increased oxidative stress in liver mitochondria[39,41].

Excessive fatty acids might be used as an alternative pathway to produce mitochondrial injury rather than the mitochondrial β-oxidation pathway. These possible routes of injury include the peroxisomal and microsomal oxidation systems[42]. Alternative fatty acid oxidation systems produce more hydrogen peroxide and thus may contribute to oxidant stress. Increased cytochrome P450 2E1 expression and induction have been well-established previously in both murine SH and human NASH studies.

In a very recent study, researchers developed a novel model which suggested maternal fat intake contributes toward the NAFLD progression in adult offspring; mediated through impaired hepatic mitochondrial metabolism and up-regulated hepatic lipogenesis[26]. Large-scale gene expression study, particularly a whole genome array analysis, is a very powerful technique which was used to measure the differences between the samples in this model[26]. This technique was able to measure the mRNA expressed in the tissue analyzed, assessing parameters such as relative expression of genes involved in inflammation, oxidative stress, lipogenesis, and beta-oxidation.

CONCLUSION

One of the areas for ongoing research is the understanding of how much calorie intake and composition of the diet affect the development of NASH. In conclusion, although these animal NAFLD models are already in use and further improve our knowledge, the underlying mechanisms in NAFLD pathogenesis need further research.

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ORIGINAL ARTICLE

Cost-utility of molecular adsorbent recirculating system treatment in acute liver failure

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Abstract

AIM: To determine the short-term cost-utility of molecular adsorbent recirculating system (MARS) treatment in acute liver failure (ALF).

METHODS: A controlled retrospective study was conducted with 90 ALF patients treated with MARS from 2001 to 2005. Comparisons were made with a historical control group of 17 ALF patients treated from 2000 to 2001 in the same intensive care unit (ICU) specializing in liver diseases. The 3-year outcomes and number of liver transplantations were recorded. All direct liver disease-related medical expenses from 6 mo before to 3 years after ICU treatment were determined for 31 MARS patients and 16 control patients. The health-related quality of life (HRQoL) before MARS treatment was estimated by a panel of ICU doctors and after MARS using a mailed 15D (15-dimensional generic health-related quality of life instrument) questionnaire. The HRQoL, cost, and survival data were combined and the incremental cost/quality-adjusted life years (QALYs) was calculated.

RESULTS: In surviving ALF patients, the health-related quality of life after treatmeant was generally high and comparable to the age- and gender-matched general Finnish population. Compared to the controls, the average cost per QALY was considerably lower in the MARS group (64732€ *vs* 133858€) within a timeframe of 3.5 years. The incremental cost of standard medical treatment alone compared to MARS was 10928€, and the incremental number of QALYs gained by MARS was 0.66.

CONCLUSION: MARS treatment combined with standard medical treatment for ALF in an ICU setting is more cost-effective than standard medical treatment alone.

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Key words: Albumin dialysis; Cost-efficiency; Health-related quality of life; Quality-adjusted life year

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INTRODUCTION

Since its introduction in 1993[1,2], the molecular adsorbent recirculating system (MARS) has been used in the treatment of both acute liver failure (ALF) and acute-on-chronic liver failure (AOCLF). The MARS device is an extracorporeal albumin dialysis apparatus that removes both albumin-bound and water-soluble toxins from the patient's blood, enabling native liver regeneration and allowing time to locate a suitable organ for liver transplantation (Ltx)[3-5].

Numerous studies have documented the favorable effects of MARS treatment on clinical and laboratory parameters[4,6-9] and survival[10-13]. However, only three small non-randomized studies[14-16] have focused on the cost-utility of MARS treatment in AOCLF and the health-related quality of life (HRQoL) of MARS-treated AOCLF patients. Currently, there are no studies on the HRQoL or cost-utility of MARS treatment in ALF patients.

In assessing therapy utility, the subjective feelings of the patient should be taken into account in addition to health benefits (e.g. survival) and cost. To ensure that limited resources are utilized in an ethical manner, the impact of a given treatment on the future HRQoL of the patient must be considered[17]. The effectiveness of a given treatment should be assessed by considering the treatment's impact on both the length and quality of life, which can be combined into the measure quality-adjusted life years (QALYs). In the cost-utility analysis, the QALYs gained by a given treatment are used as the measuring units of efficacy. Thus, the QALYs and cost/QALY-ratios can be used to compare the different treatments in terms of length of life and quality of life. Currently, there is no consensus as to how much a QALY gained can cost, but a 50 000€ threshold has been suggested[17].

The aim of this study was to determine the short-term (3.5 years) cost-utility of MARS treatment in ALF patients.

MATERIALS AND METHODS

Patients

This study included 90 ALF patients treated with MARS from May 2001 to October 2005 and a historical control group of 17 consecutive ALF patients treated from March 2000 to April 2001. ALF was defined as a rapid deterioration of hepatic synthetic function with or without encephalopathy and no previous history of liver disease[18].

All patients were treated in the same intensive care unit (ICU) specializing in liver disease at the Helsinki University Hospital and according to the same main principles of standard medical therapy (SMT). The SMT in our ICU and the operational principles of the MARS device were reported previously[3-5]. Our liver ICU is the only Ltx center in Finland, and all critical ALF patients are referred to our unit for treatment and transplantation evaluation. The indications for MARS treatment and the treatment protocols are summarized in Table 1. The 3-year survival and the need for Ltx were determined in all patients.

Economic evaluation

In the cost-utility analysis, effectiveness was measured as QALYs gained. The costs and outcomes of MARS treatment were compared with those of SMT in the control group over a 3-year time horizon from the perspective of the health care provider. For this comparison, a deterministic decision model was developed using TreeAge Pro HealthCare software (TreeAge, Williamstown, MA, USA). The model was used to combine the data on costs and effectiveness and to incorporate data variability and uncertainty into the analysis (Figure 1). The pathways presented in Figure 1 are mutually exclusive sequences of events, and the expected values are based on the pathway values weighted by the pathway probabilities. The probabilities show the proportion of the patient cohort that is expected to experience the event at any particular point in the tree.

The incremental cost-effectiveness ratio (ICER) was determined by dividing the difference in costs by the difference in QALYs. Both costs and QALYs were discounted 5% as currently recommended in the Finnish guidelines for pharmacoeconomic evaluation. The base-case analysis estimated the cost-effectiveness based on the mean values from the data in the simplified model structure. The effect of model input parameter uncertainty on the results was tested in a one-way and probabilistic sensitivity analysis.

Direct costs

From the year 2000 onwards, the Helsinki University Central Hospital district began to use the clinical patient-administration database Ecomed® for registering all treatment costs. In this study all direct medical costs were obtained from the Ecomed®-database (Datawell Ltd., Espoo, Finland). The total costs included all relevant liver disease-related expenses incurred at the Helsinki University Hospital. All costs incurred between 6 mo before the first MARS treatment (or liver ICU admission in the control group) and 3 years after the treatment were included. Complete cost data were available only for those patients who were living (31 MARS and 16 control patients) and received all hospital care in the catchment area of the Helsinki and Uusimaa Hospital District. Therefore, patients referred from outside the catchment were excluded from the cost analysis. Direct non-medical costs (transportation, domestic help, productivity costs due to absences from work, *etc.*) were not included. The mean total cost within the specified time period was used in the base-case analysis. All costs in Euros were inflated to the 2006 price level.

Table 1 MARS treatment protocols for acute liver failure in Finland

Etiology	MARS treatment initiation criteria	Treatment protocol
Acute liver failure	Rapid deterioration of hepatic synthetic function and clinical condition despite conservative standard medical therapy And one of the following criteria: (1) Ingestion of a lethal dose of a known hepatotoxin (e.g. *Amanita*, paracetamol) (2) The patient fulfills the criteria for highly urgent liver transplantation	22-h sessions daily until: (1) Native liver recovers (2) Suitable transplant organ is found (3) Irreversible multi-organ damage occurs

MARS: Molecular adsorbent recirculating system.

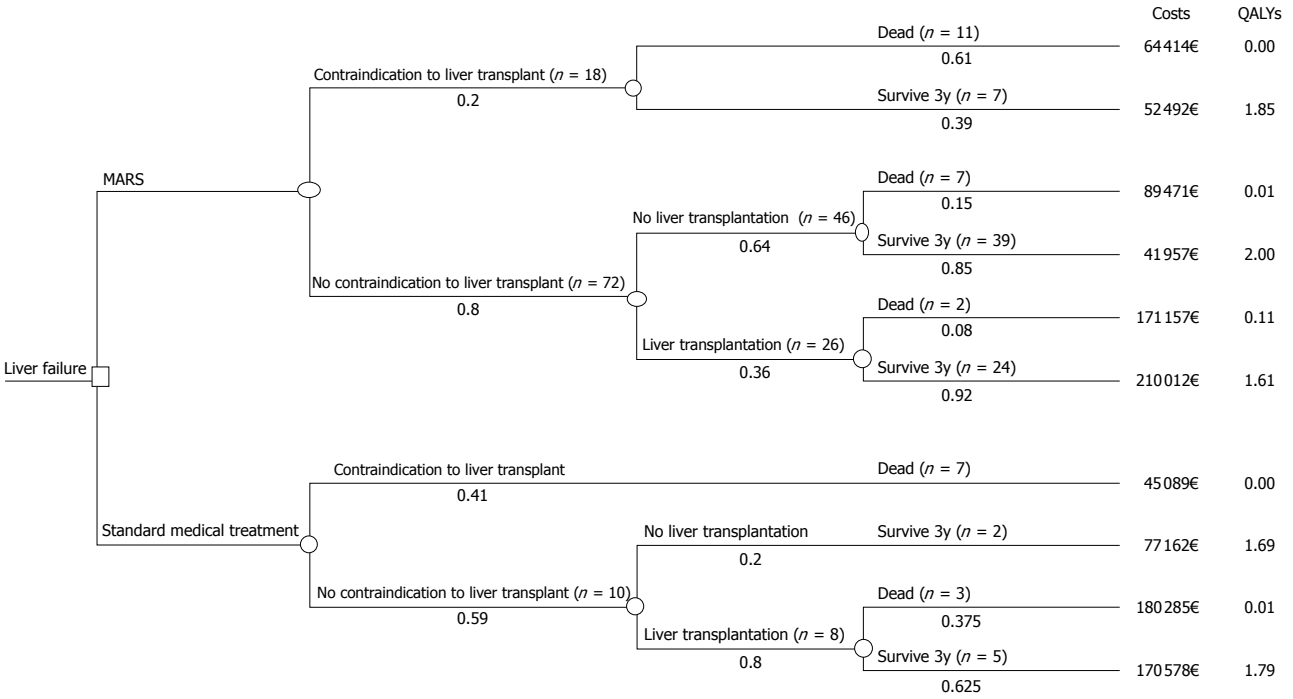


Figure 1 A deterministic decision model. In both treatment arms, the patient cohort was divided based on contraindication *vs* no contraindication to liver transplantation (Ltx) and then on the basis of Ltx *vs* no-Ltx. The "survive 3y" branches include all patients who survived at least three years after the first treatment, and the "death" branches include all patients who died within those three years. The pathway probabilities i.e. the proportion of the cohort that is expected to experience the event, are shown under each branch and were calculated based on real patient data. MARS: Molecular adsorbent recirculating system; QALY: Quality-adjusted life year.

HRQoL

The HRQoL was measured by the 15D (15-dimensional generic health-related quality of life instrument)[19-21], which is a generic, self-administered questionnaire for adults using criteria related to 15 dimensions: moving, seeing, hearing, breathing, sleeping, eating, speech, eliminating, usual activities, mental function, discomfort and symptoms, depression, distress, vitality, and sexual activity. For each dimension, the patient chooses one of five levels that best describes his/her current state of health. A set of utility weights is used to generate a single index number, the 15D score, which ranges from 0 to 1 (1 = full health, 0 = dead)[22]. For most of the important properties (i.e. reliability, content validity, discriminatory power, and responsiveness to change), the 15D is at least equal to other similar HRQoL instruments, such as the EQ-5D, SF-6D, HUI3, and AqoL[19-21,23-25].

Most ALF patients admitted to our liver ICU are seriously ill and encephalopathic, if not unconscious, and thus unable to fill out the HRQoL questionnaires. Therefore, we used expert opinion to assess the pre-treatment HRQoL. Three ICU doctors separately and retrospectively estimated the HRQoL of 30 ALF patients using the 15D instrument and the patients' clinical documents. All patients were divided into five groups according to their pre-treatment encephalopathy grade, which was represented as a number from 0 to 4. The HRQoL was assessed in all patients who were conscious and not intubated. The HRQoL was evaluated for six randomly selected non-intubated patients from each encephalopathy grade 0-3, and their average 15D score was assumed to represent the approximate HRQoL of the entire group (Table 2). Unconscious and intubated patients, which included some encephalopathy grade 3 patients and all encephalopathy grade 4 patients, received a 15D score of 0.0162[22].

In July 2007, the 15D questionnaire was sent to the 68 MARS-treated ALF patients who were still alive (one patient was not found). Two reminder letters were sent to those who did not return the first questionnaire. In total, 79% (54/68) completed the post-treatment 15D questionnaire; 37% of these patients (20/54) had undergone Ltx. At the time of the survey, the time elapsed after the first MARS treatment varied among individual patients

Table 2 Mean pre-treatment 15D scores in different encephalopathy grade groups (mean ± SD)

Pre-treatment encephalopathy grade	15D score
0	0.532 ± 0.151
1	0.461 ± 0.188
2	0.403 ± 0.177
3	0.079 ± 0.077
4	0.016

The average age-matched 15D score of the Finnish population is 0.92, and the standardized 15D score for unconscious/intubated patients is 0.016[22].

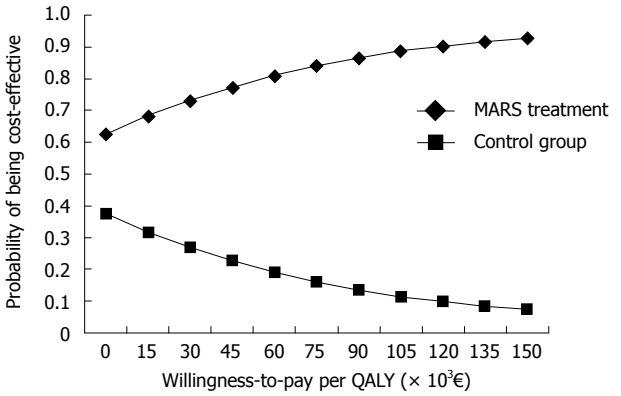


Figure 2 The cost-effectiveness acceptability curve for MARS *vs* standard medical therapy in the historical control group in acute liver failure.

(median 49 mo, range 22-72 mo). Thus, a 15D score 3 years after the first MARS treatment was individually estimated for each patient using linear regression analysis with the patient's age, time since MARS treatment, and the etiology of liver failure as explanatory variables.

The mean 15D scores and resulting QALYs were calculated separately for each pathway in the decision tree (Figure 1). For patients who died within the time horizon, QALYs were calculated based on survival, assuming the 15D score declined linearly from the baseline value to zero at the time of death.

Sensitivity analysis

The effect that model parameter uncertainty had on the results was examined first in a one-way analysis and further in a probabilistic sensitivity analysis. In the one-way sensitivity analysis, the discount rate, the mean cost and QALYs of both the MARS and control groups, the probability of Ltx, and survival rates varied. For the probabilistic sensitivity analysis, probability distributions for tree probabilities, cost, and utility input parameters were estimated. The results of the probabilistic sensitivity analysis are presented as a cost-effectiveness acceptability curve (Figure 2). Probability and utility values are represented by beta distributions (limiting values between 0 and 1 for utility values), the parameters of which were estimated from the patient data. For cost estimates, a gamma distribution was determined. Because the actual patient level cost data were limited, regression analysis was used to estimate costs for all patients. Sex, Ltx and its contraindication, and the number of MARS treatments received were used as explanatory variables in the regression analysis.

Table 3 Baseline data for the MARS and control groups

	MARS group	Historical control group	*P* value
Pre-treatment demographic & clinical data			
Number of patients	90	17	
Age (yr)	45 (14-81)	42 (21-72)	0.714
Sex (male)	37 (41%)	7 (41%)	0.996
Body mass index (kg/m^2)	26 (17-40)	26 (21-37)	0.372
MARS sessions/patient	2 (1-9)	0	
Mechanically ventilated	30 (33%)	6 (35%)	0.875
Vasoactive infusion used	27 (30%)	9 (56%)	0.041
Renal insufficiency	29 (32%)	7 (41%)	0.474
MELD-score	31 (5-50)	30 (19-51)	0.225
Mean encephalopathy grade	1.7 ± 1.6	2.1 ± 1.7	0.335
Contra-indication to Ltx prior to treatment	11 (12%)	4 (24%)	0.253
Became untransplantable during treatment	7 (8%)	3 (18%)	0.195
Number of transplanted patients	26 (29%)	8 (47%)	0.162
Pre-treatment key laboratory values			
Platelets (× 10^9/L)	138 (11-410)	142 (51-448)	0.919
NH_4 ion (μmol/L)	68 (8-512)	90 (14-241)	0.559
Bilirubin (μmol/L)	215 (4-761)	381 (38-880)	0.057
Creatinine (μmol/L)	75 (35-1318)	78 (38-275)	0.946
FV (%)	32 (5-142)	38 (7-71)	0.865

All values are expressed as median (range) or as number of patients (%). Hepatic encephalopathy grade is presented as mean ± SD. MELD score: Mean end-stage liver disease score; Ltx: Liver transplantation; FV: Coagulation factor V.

Statistical analysis

All data were analyzed using SPSS for Windows version 15.0 (SPSS, Inc., Chicago, IL, USA). The Wilcoxon signed-rank test, the Mann-Whitney *U*-test, Pearson's χ^2, and Fisher's exact tests were used when appropriate. $P \leqslant 0.05$ was considered significant, and a $\geqslant 0.03$ (absolute value) difference in the HRQoL scores was considered clinically important[26].

RESULTS

Characteristics of the study population

The demographic and clinical characteristics of the MARS-treated and control ALF patients did not differ significantly, with the exception of a need for vasoactive infusion (Table 3). The grade of encephalopathy before treatment was also comparable between the two groups. The etiological distribution of ALF patients differed between the groups ($P = 0.002$). Control patients had mostly ALF of unknown etiology (65%), whereas the majority of MARS-treated patients (57%) had ALF due to known toxicity (e.g. paracetamol or other drugs).

Outcome and 3-year survival

The percentage of transplanted patients was lower (29% *vs* 47%, $P = 0.14$), and the percentage of patients who survived 3 years after treatment was higher (78% *vs* 41%, $P = 0.002$) in the MARS-treated group compared to controls.

Table 4 The survival, cost, and HRQoL data of MARS-treated and control patients

	Subgroups according to outcome	3-yr survival			Cost (€)				HRQoL		
		n	%	Mean (d)	*n*	Mean	Min	Max	*n*	Pre-treatment mean	Post-treatment mean
MARS group	All ALF patients	90	78		31	79745	11961	370573	90	0.30	0.70
	Alive at 3 yr	70			24	78724	11961	370573	70	0.34	0.89
	Dead	20		63	7	83243	32496	171157	20	0.15	0.00
	Contraindication to Ltx	18	39								
	Alive at 3 yr	7			4	52492	30325	112585	7	0.38	0.85
	Dead	11		14	5	64414	32496	95666	11	0.13	0.00
	No contraindication - no Ltx	46	85								
	Alive at 3 yr	39			15	41957	11961	137235	39	0.40	0.93
	Dead	7		110	1	89471			7	0.09	0.00
	Transplanted	26	92								
	Alive at 3 yr	24			5	210012	96984	370573	24	0.22	0.84
	Dead	2		169	1	171157			2	0.47	0.00
Control group	All ALF patients	17	41		16	105820	16862	262481	17	0.27	0.36
	Contraindication to Ltx		0								
	Dead	7		9	7	45089	17591	105917	7	0.27	0.00
	No contraindication - no Ltx		100								
	Alive at 3 yr	2			2	77162	16862	137462	2	0.27	0.85
	Transplanted		63								
	Alive at 3 yr	5			4	170578	117444	262481	5	0.32	0.87
	Dead	3		28	3	180285	129120	250772	3	0.19	0.00

HRQoL: Health-related quality of life; ALF: Acute liver failure.

Table 5 Base case results for MARS

	Cost (€)	Incremental cost (€)	QALYs	Incremental QALYs	Cost per QALY (€)	Incremental cost per QALY
MARS	93214		1.44	0.66	64732	MARS dominates SMT
Control group (SMT only)	104142	10928	0.78		133858	

SMT: Standard medical therapy; QALY: Quality-adjusted life year.

A similar survival benefit favoring MARS treatment compared to controls was noted in both transplanted (92% *vs* 63%, $P = 0.072$) and non-transplanted patients (72% *vs* 22%, $P = 0.006$).

Costs

The total mean direct medical costs related to liver disease were 79745€ for the MARS group and 105820€ for the controls (Table 4). The total mean direct medical costs for transplanted and non-transplanted MARS and control patients are shown in Figure 3 and the expected costs weighted by the pathway probabilities are shown in Table 5.

In all patient groups, most of the costs were incurred within a year of the first MARS or standard medical treatment. The costs during the second and third years were negligible. In all groups, the highest costs were observed in transplanted patients.

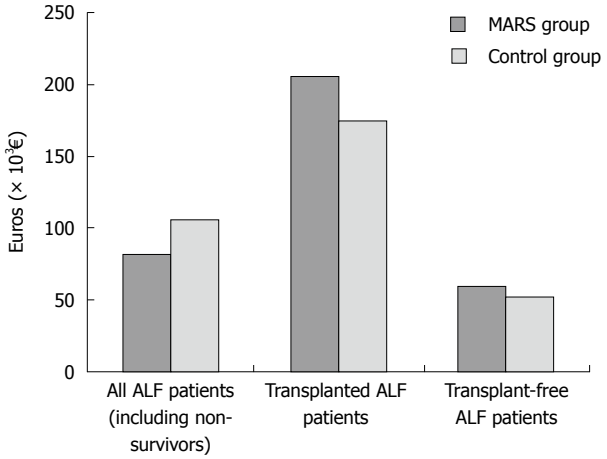


Figure 3 The 3.5-year mean overall direct medical costs per patient in the MARS and control groups. ALF: Acute liver failure.

HRQoL and QALYs

The pre- and post-treatment 15D scores are shown in Figure 4 and Table 4. The estimated HRQoL for all patients prior to treatment was very low compared to an age-standardized reference population in Finland (0.30 *vs* 0.92)[27]. The highest post-treatment 15D scores were observed in MARS-treated patients who did not have a contraindication to Ltx and recovered without transplantation. The same patients also experienced the highest mean number of QALYs (2.00, within the 3-year follow-up). The groups with the smallest mean number of QALYs were patients with a contraindication to Ltx and those who died within 3 years of treatment.

Cost-utility analysis

In the base-case analysis, the MARS group strongly domi-

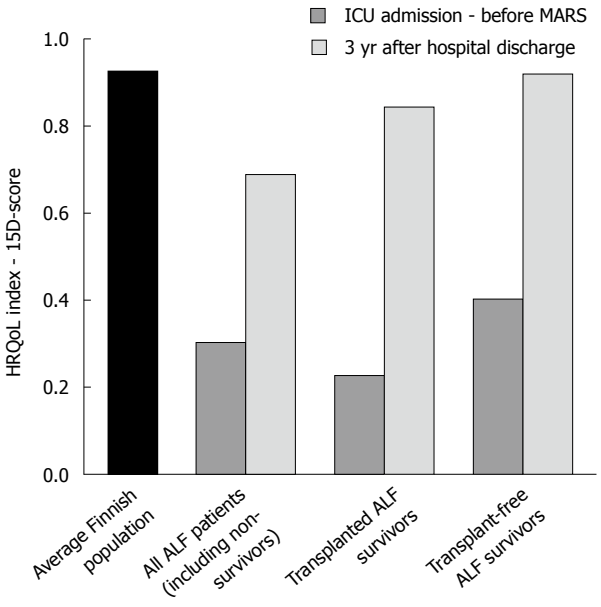


Figure 4 The 15D-score before and 3 years after MARS treatment. HRQoL: Health-related quality of life.

nated the control group receiving only SMT; MARS treatment was both less costly and more effective than SMT in ALF patients (Table 5). Using the 3-year time horizon, the expected outcome of MARS patients was 1.44 QALYs and the expected costs were 93 214€. The corresponding figures for the controls were 0.78 and 104 142€, respectively. Compared to MARS, the incremental cost of SMT in the control group was 10 928€. The incremental number of QALYs gained by MARS was 0.66.

Sensitivity analysis

MARS remained the dominant strategy throughout most of the one-way sensitivity analyses. Neither increasing the proportion of patients with a contraindication to Ltx in the MARS group nor varying the survival rates of patients after Ltx or MARS-treated patients with a contraindication, mitigated the dominance of MARS treatment. MARS also remained the dominant strategy throughout QALY estimate variation in the one-way sensitivity analysis. Using a discount rate of 0%, 3%, or 5% did not eliminate the dominance.

The results were more sensitive to variation in the cost estimates. In the one-way sensitivity analysis, increasing the costs for MARS-treated patients who had no contraindication and survived three years with Ltx removed the dominance of MARS and resulted in an ICER of 63 206€ per QALY gained.

The proportion of liver disease with unknown etiology was significantly higher in the control group (P = 0.002), which can lead to a higher probability of Ltx. We varied the probability of Ltx in the MARS group from 0.89 (observed in controls) to 0.36 (observed in the MARS-treated group); throughout which, MARS remained the more effective alternative. However, when the probability of Ltx in the MARS group was increased to 0.42 or higher, the expected cost of MARS exceeded that of the control group receiving SMT, and its dominance was eliminated. When the probability of Ltx in the MARS group was set to the same level as the control group (0.89), the ICER was 98 686€ per QALY gained.

The cost-effectiveness acceptability curve (Figure 2) of MARS *vs* controls showed the probability that MARS is cost-effective given a range of willingness-to-pay thresholds per QALY gained. If the decision-maker is willing to pay 50 000€ per QALY, the probability of MARS being cost-effective is 78%. Furthermore, the probability of MARS being cost-effective is 95% if the willingness-to-pay threshold is 200 000€ per QALY.

DISCUSSION

To our knowledge, there are no previous studies evaluating the cost-utility of MARS treatment in ALF patients. We found that MARS treatment was both less costly and more effective than SMT in ALF. Compared to the controls, the average cost per QALY was significantly lower in the MARS group (64 732€ *vs* 133 858€), mainly due to the significantly higher 3-year overall survival rate and fewer transplantations in the MARS group.

The average overall 3.5-year costs associated with the treatment of ALF were substantial with or without MARS treatment due to the long ICU stay and, in some cases, Ltx costs. The fewer Ltx in the MARS group resulted in reduced overall average costs compared to conservative treatment. Because the costs associated with the Ltx procedure are high, any intervention that decreases the number of transplantations is bound to have a profound effect on the total expense.

As reported previously[28,29], we found that, even though the HRQoL of surviving transplanted patients was generally very good, it was still somewhat lower than that of a person in the age-standardized general population (0.84 *vs* 0.92)[27]. In ALF patients who recovered without Ltx, the HRQoL after treatment was similar to that of the age-standardized Finnish reference population (0.93 *vs* 0.92)[27].

Only a handful of HRQoL and cost-effectiveness studies have been completed on MARS patients, and all have been limited to AOCLF patients treated in the same center[14-16]. Until now, MARS-treated ALF patients have not been evaluated in terms of total cost and QALYs, possibly owing to the rarity of the condition, which makes it difficult to enroll enough patients for statistical analysis. Furthermore, comparing results from different studies can be difficult because transplant organ availability varies, and centers may have different criteria for MARS treatment. In addition, the heterogeneity of the etiology of ALF in different countries markedly affects survival rates and the percentage of patients who may experience native liver recovery[30,31].

The most recent cost-utility evaluation of MARS treatment included 79 alcohol-related AOCLF patients[14]; their 3-year survival was 52% with mean direct medical costs of 40 032€ and an ICER of 31 448€ per life-year gained. However, transplanted patients and those with serious co-

morbidities were excluded from this analysis, which had a huge impact on the total costs. Another study focused on the impact of MARS treatment on the hospitalization costs and 1-year survival of cirrhotic AOCLF patients[32]; the in-hospital cost for surviving MARS-treated patients was $32 036, $4000 less than in the control group.

The main limitation of this study was the nonrandomized design. Also, due to patient incapacitation, the pretreatment HRQoL was retrospectively assessed by an expert panel rather than by the patients themselves. Studies have shown that the subjective feelings of a patient, such as pain or HRQoL, are usually misjudged or underestimated by the attending doctors, other treatment staff, or even close relatives[33,34]. Another factor affecting the positive outcomes of MARS-treated ALF patients in this study was the uneven distribution of liver failure etiologies between treatment groups. The prognosis and need for Ltx is strongly related to the underlying cause of ALF[30]; the MARS treatment group was at an advantage in that it included many patients with a good prognosis compared to controls. This predisposition must be taken into account when interpreting the results of this study. The sensitivity analysis was used to estimate the effect of this bias, and it seems that even if both the MARS and control groups had a similar distribution of etiologies, MARS treatment would remain the more effective strategy, although more expensive in terms of total cost.

One must bear in mind that this study dealt with a 3.5-year time window, thus severely underestimating the QALYs to be gained over the remaining lifetime. Alternatively, we could have extrapolated the expected lifetime costs and outcomes; however, this would have necessitated further assumptions and resulted in greater uncertainty.

In conclusion, cost-utility analysis found that MARS treatment plus SMT is both less costly and more effective than SMT alone in ALF patients. Although some uncertainty exists in the model input parameters, probabilistic sensitivity analysis showed that the probability of MARS being cost-effective is higher than that of SMT alone.

COMMENTS

Background

The molecular adsorbent recirculating system (MARS) device is an extracorporeal albumin dialysis apparatus which can be used to treat liver failure patients. MARS removes both albumin-bound and water-soluble toxins from the patient's blood, enabling native liver regeneration and allowing time to locate a suitable organ for liver transplantation. Numerous studies have documented the favorable effects of MARS treatment on clinical and laboratory parameters and survival. However, only a few small non-randomized studies have focused on the cost-utility of MARS treatment in patients with acute-on-chronic liver failure.

Research frontiers

The cost-utility of the MARS treatment and its impact on the health-related quality of life in acute liver failure patients has never been investigated previously.

Innovations and breakthroughs

The results from the current study suggest that MARS treatment is both less costly and more effective than standard medical therapy in the treatment of acute liver failure patients. Compared to the controls, the average cost per quality-adjusted life year (QALY) was significantly lower in the MARS group, mainly due to the significantly higher 3-year overall survival rate and fewer transplantations in the MARS group. The authors also found that in MARS-treated acute liver failure patients who recovered without liver transplantation, the health-related quality of life after treatment was similar to that of the age-standardized Finnish reference population.

Applications

The cost-utility analysis suggests that MARS treatment plus standard medical therapy is both less costly and more effective than standard medical therapy alone in acute liver failure patients. Therefore, the additional costs which are associated with the use of the MARS machine seem justifiable in this patient group.

Peer review

This paper deals with the important topic of the cost-utility of molecular adsorbent recirculating system treatment in acute liver failure. The report is concise and informative.

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ORIGINAL ARTICLE

Apoptotic activity of caged xanthones from *Garcinia hanburyi* in cholangiocarcinoma cell lines

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Abstract

AIM: To investigate the growth inhibitory mechanism of four caged xanthones from *Garcinia hanburyi* in cholangiocarcinoma (CCA) KKU-100 and KKU-M156 cells.

METHODS: Four caged xanthones, selected on the basis of their anticancer potency and chemical structure diversities (i.e. isomorellin, isomorellinol, forbesione and gambogic acid) were used in this study. Growth inhibition of these caged xanthones was determined using the sulforhodamine B assay. Induction of apoptosis was assessed by observing cell morphology, ethidium bromide and acridine orange staining and DNA fragmentation assay. Levels of apoptotic-related gene and protein expressions were determined by a real-time reverse transcriptase polymerase chain reaction and Western blotting analysis, respectively.

RESULTS: The compounds were found to inhibit growth of both cell lines in a dose-dependent manner and also showed selective cytotoxicity against the cancer cells when compared with normal peripheral blood mononuclear cells. Growth suppression by these compounds was due to apoptosis, as evidenced by the cell morphological changes, chromatin condensation, nuclear fragmentation, and DNA ladder formation. At the molecular level, these compounds induced down-regulation of Bcl-2 and survivin proteins with up-regulation of Bax and apoptosis-inducing factor proteins, leading to the activation of caspase-9 and -3 and DNA fragmentation. The functional group variations did not appear to affect the anticancer activity with regard to the two CCA cell lines; however, at a mechanistic level, isomorellinol exhibited the highest potency in increasing the Bax/Bcl-2 protein expression ratio (120 and 41.4 for KKU-100 and KKU-M156, respectively) and in decreasing survivin protein expression (0.01 fold as compared to control cells in both cell lines). Other activities at the molecular level indicate that functional groups on the prenyl side chain may be important.

CONCLUSION: Our findings for the first time demonstrate that four caged xanthones induce apoptosis in

CCA cells which is mediated through a mitochondria-dependent signaling pathway.

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Key words: *Garcinia hanburyi*; Caged xanthones; Human cholangiocarcinoma cell lines; Apoptosis

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INTRODUCTION

Cholangiocarcinoma (CCA) is a malignant tumor arising from bile duct epithelial cells[1], characterized by a poor prognosis and unresponsive to conventional chemotherapeutic agents. The increasing global incidence of this tumor underscores the need for novel and effective therapeutic agents for CCA[2,3].

Much attention has been focused on natural products as potential sources of novel anticancer drugs over the decades[4]. An emerging class of compounds, being potent antiproliferatives as well as having anticancer and anti-tumor activities against mammalian cancer cell lines, is caged xanthones. Recent work by our and other research groups has identified a tropical plant, *Garcinia hanburyi* (*G. hanburyi*) Hook.f. (family Guttiferae), used as a purgative and externally for infected wounds in Thai traditional medicine[5], as a rich source of these compounds[6-11]. In addition, gambogic acid, derived from the gamboges resin of *G. hanburyi,* has been shown to induce apoptosis in human gastric carcinoma cells[9]. These data suggest that these caged xanthones might be effective as chemotherapeutic agents and that they warrant further study of the underlying mechanisms of their anticancer activities.

Our interest in searching for agents for the effective treatment of CCA led us to test a series of caged xanthones from *G. hanburyi*[10] against two human CCA cell lines. Among the compounds tested, isomorellin, isomorellinol, forbesione and gambogic acid (Figure 1) exhibit promising IC_{50} values against both cell lines in comparison with the standard drug doxorubicin. In view of their anticancer potency and chemical structure diversity, these four caged xanthones were selected for a more in-depth study. In order to further delineate the underlying mechanisms, the apoptotic action of these compounds was investigated in detail. The results are the main focus of this communication.

MATERIALS AND METHODS

Reagents and cell culture

Four caged xanthones, i.e. isomorellin, isomorellinol, forbesione and gambogic acid, were isolated from *G. hanburyi* Hook.f. (family Guttiferae) using bioassay-directed fractionation[10]. The KKU-100 and KKU-M156 cells were isolated from Thai CCA patients and the original characterization of these cell lines has been described previously[12,13]. Human peripheral blood mononuclear cells (PBMCs) were freshly isolated using the standard Ficoll-hypaque gradient centrifugation method and used as normal control cells[14]. Cells were grown in RPMI 1640 (GIBCO BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/mL of penicillin and 100 μg/mL streptomycin (GIBCO BRL) at 37℃ in a humidified incubator containing 5% CO_2.

Cell proliferation assay

For the cell proliferation assay, 1.9×10^4 cells/well were seeded in 96-well microtitre plates and incubated for 24 h. After treatment for 72 h with 0-8.8 × 10^4 μmol/L per well for the caged xanthones, 0.04, 0.4, 4, 40 and 400 μmol/L for doxorubicin (Boryung Pharmaceutic Co. LTD, Korea) as a reference compound and DMSO as the solvent-control cells, cell growth was measured using the sulforhodamine B (SRB) assay[15].

Morphological examination

KKU-100 and KKU-M156 cells (1×10^6) were grown in a 25 cm^2 flask at 37℃ for 24 h, and treated with 2 × IC_{50} concentration of each caged xanthone for 48 h. Morphological changes occurring in the cells were observed under bright field inverted Nikon microscope. For nuclear staining, cells (1.9×10^3 cells/well) were grown in 96-well microtitre plates at 37℃ for 24 h, and treated with 2 × IC_{50} concentration of each caged xanthone for 24, 36 and 48 h. The treated cells were stained with 14 μL of 100 μg/mL ethidium bromide/acridine orange (EB/AO) mixture (Sigma Chemical, St. Louis, MO) and observed under a Nikon fluorescent microscope. Apoptotic cells with condensed chromatin or fragmented chromatin were counted and expressed as a percentage from a total of 500 cells each[16].

DNA fragmentation assay

The isolation of fragmented DNA was carried out according to the procedure of Herrmann *et al*[17] with some modifications. Briefly, after culturing for 24 h and starving in medium containing 0.5% FBS for 24 h, cells (1×10^6) were treated with DMSO or 2 × IC_{50} concentrations of the caged xanthones for 24 and 36 h. After extraction, DNA in cell lysate was purified by QIAamp DNA Blood Mini Kit (QIAGEN, Germany) according to the manufacturer's protocol. The DNA fragments were precipitated with ethanol, re-suspended in 50 μL of TE buffer, and analyzed by electrophoresis.

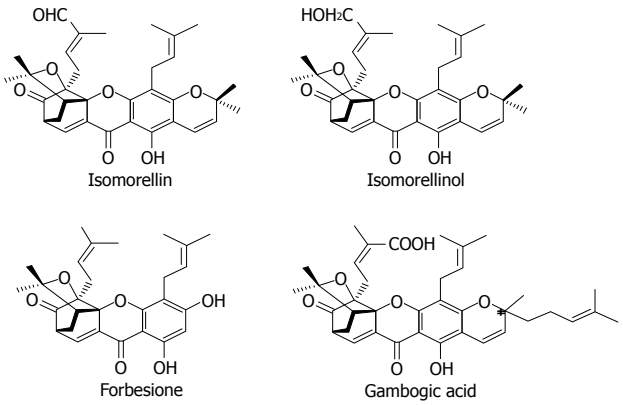


Figure 1 Chemical structure of four caged xanthones.

RNA extraction, reverse transcription and quantitative real-time polymerase chain reaction

Cells were treated with 2 × IC_{50} concentrations of the caged xanthones for 0, 6, 12, 24 and 48 h. Total RNA was isolated from the treated and control cells using TRIzol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. The reverse transcription reaction was performed as described[18].

Real-time polymerase chain reaction (PCR) was performed according to the procedure of Namwat *et al*[18]. Real-time PCR of Bcl-2, Bax, survivin and GAPDH were performed in a 20 μL PCR reaction mixture containing first strand cDNA, 5 pmol of each primer, and 10 μL of 2 × SYBR Green PCR Master Mix (Gene Systems Co., Ltd, USA), employing an ABI 7500 Real-time PCR system (Applied Biosystems, Foster City, CA). The PCR primers (Bioservice Unit, Bangkok, Thailand) are shown in Table 1. The sequences of these PCR products were obtained by direct sequencing. Each amplicon was then cloned into the pGEM®-T vector (Promega, Madison, WI, USA) in order to generate standard curves for target cDNA. The mRNA level is reported as the ratios of the copy numbers of target cDNA to GAPDH cDNA. The ratio of fold change in gene expression level between the treated and control cells was determined to identify the up- or down-regulation of gene expression.

Protein extraction and Western blotting analysis

After treatment with DMSO or 2 × IC_{50} concentrations of the caged xanthones for 0, 12, 24 and 48 h, protein extraction and Western blotting were performed as described previously[19]. After blocking, the membranes were incubated at 4℃ overnight with the following antibodies: Bcl-2, Bax, survivin, apoptosis-inducing factor (AIF) (Santa Cruz Biotechnology, CA) or procaspase-9 and -3, activated caspase-9 (Cell Signaling, Beverly, MA) or activated caspase-3, β-actin (Sigma Chemical, St. Louis, MO). The secondary antibodies were horseradish peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG antibodies (Santa Cruz Biotechnology). Protein bands were detected by an enhanced chemiluminescence kit (Pierce Biotechnology, Rockford). The intensity of the protein bands was quantified using Scion Image software.

Statistical analysis

Data were expressed as mean ± SE. Comparisons between untreated control cells and the caged xanthone-treated cells were made using Student's *t* test. Differences were considered significant at $P < 0.05$. All analyses were performed using SPSS version 10.0 (SPSS Inc., USA).

RESULTS

Anti-proliferative effects of four caged xanthones on CCA cell lines

The treatment of KKU-100 and KKU-M156 cells with four caged xanthones markedly inhibited cell growth in a dose-dependent manner (Figure 2). The IC_{50} values of these caged xanthones and doxorubicin are shown in Table 2. The anticancer potencies of these compounds are comparable to doxorubicin in both cell lines. These caged xanthones induced growth inhibition in the CCA cell lines about 4 to > 8 × 10^6 times greater than that for normal PBMCs (Table 2). These results indicate that these compounds possess considerable potential for further development.

Apoptosis induction by four caged xanthones in CCA cell lines

In both cell lines, treatment with four caged xanthones resulted in cell shrinkage, rounding and membrane blebbing, thus taking on the typical characteristics of apoptotic cells (Figure 3C and D); whereas no effect was observed with the vehicle control cells (Figure 3A and B). Using EB/AO staining, both cell lines showed nuclei with homogeneous chromatin distribution under vehicle-control conditions (Figure 3E and F). In the treated cells, characteristic apoptotic features such as chromatin condensation and nuclear fragmentation were observed (Figure 3G and H). The number of apoptotic cells in both cell lines treated with four caged xanthones was significantly increased in a time-dependent manner (Table 3). Using an agarose gel electrophoresis, a typical ladder pattern of internucleosomal DNA fragmentation, a hallmark of apoptotic cell death[20], was observed in the treated cells (Figure 3I and J).

Effect of four caged xanthones on the expression of apoptosis-related genes and proteins

In both CCA cell lines treated with isomorellinol there was a significant increase in both mRNA (Figure 4A) and protein (Figure 5) of pro-apoptotic Bax, but a marked decrease in the anti-apoptotic Bcl-2 (Figure 4A and Figure 5) in a time-dependent manner leading to an enhanced Bax/Bcl-2 ratio (Figure 4B and Table 4). The same results were obtained when both CCA cell lines were treated with the other three compounds (Figure 4B and Table 4). Using Western blotting, isomorellinol was found to have the highest potency in increasing Bax/Bcl-2 ratio. At 48 h of treatment, isomorellinol increased the Bax/Bcl-2 ratio up to 120 and 41.4 in the KKU-100 and KKU-M156 cells, respectively (Table 4).

Western blotting analysis revealed that treatment of

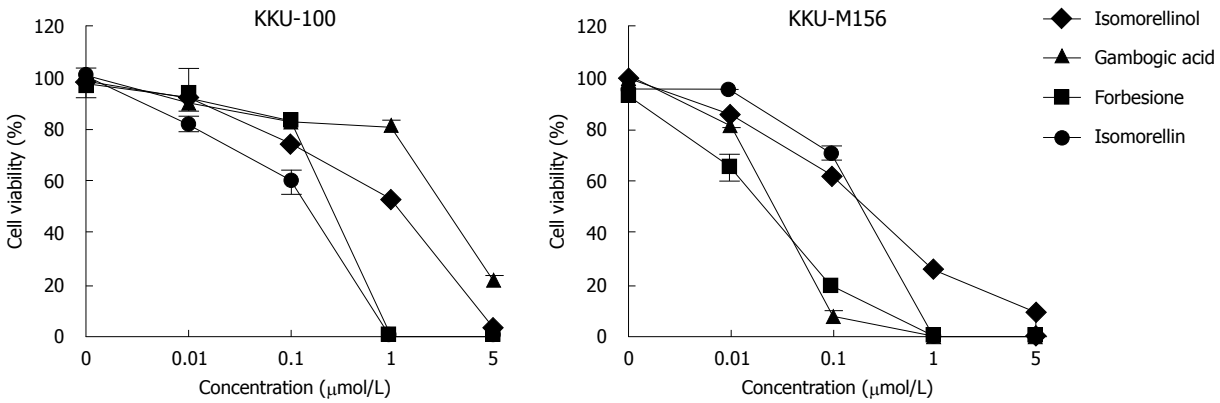


Figure 2 Antiproliferative activities of the four caged xanthones against KKU-100 and KKU-M156 cells. Cells were treated with DMSO or indicated amounts of isomorellinol, forbesione, gambogic acid and isomorellin for 72 h. The percent cell viability was determined by SRB assay. Data are expressed as mean ± SE (n = 3).

Table 1 Primer sequences used for target gene amplification

GenBank accession No.	Gene	Forward primer	Reverse primer
M14745.1	*Bcl-2*	TGGATGACTGAGTACCTGA	TGAGCAGAGTCTTCAGAGA
L22473.1	*Bax*	AACCATCATGGGCTGGA	CGCCACAAAGATGGTCA
AF077350.1	*Survivin*	AAGGCTGGGAGCCAGA	TGGCTCTTTCTCTGTCCA
BC083511	*GAPDH*	TCATCAGCAATGCCTCCTGCA	TGGGTGGCAGTGATGGCA

Table 2 Concentrations of four caged xanthones and doxorubicin causing a 50% inhibitory effect (IC_{50}) in cell proliferation

Cells	IC_{50} values (μmol/L)				
	Isomorellin	Isomorellinol	Forbesione	Gambogic acid	Doxorubicin
KKU-100	0.11 ± 0.004	2.2 ± 0.33	0.15 ± 0.007	2.64 ± 1.29	0.66 ± 0.001
KKU-M156	0.12 ± 0.005	0.43 ± 0.06	0.02 ± 0.002	0.03 ± 0.004	0.36 ± 0.001
PBMC	> 88 × 10^4	59 ± 2.9	59.5 ± 1.0	11 ± 3	ND

Data are mean ± SE of three independent experiments. ND: Not detected.

both cell lines with isomorellinol resulted in a significant reduction of procaspase-3 and -9 while increasing activated caspase-3 and -9 in a time-dependent manner (Figure 5). The treatment of both CCA cell lines with the other three compounds also showed the same results (data not shown). When the relative intensities of activated caspase-3 and -9 to β-actin of treated cells were compared to control cells (0 h), there was a multi-fold increase in the activated caspase-3 and -9 (Table 5). The high degree of multi-fold increase in activated caspase-9 was detected in the isomorellinol-treated KKU-100 and KKU-M156 cells (56 × and 58 ×, respectively), in gambogic acid-treated KKU-100 cells (78 ×) and in isomorellin-treated KKU-M156 cells (34 ×) (Table 5). All four compounds were also shown to increase the activated caspase-3 level in treated KKU-M156 cells, from 10 × to 46 × compared with the control cells (0 h), with a high degree of activated caspase-3 for isomorellinol-treated (33 ×) and gambogic acid-treated KKU-M156 cells (46.7 ×) (Table 5).

The real-time PCR and Western blotting analysis exhibited a significant time-dependent decrease in *survivin* gene (Figure 4A) and protein (Figure 5) expression in the isomorellinol-treated KKU-100 and KKU-M156 cell lines. The same results were obtained when these cell lines were treated with the three other compounds (data not shown). The multi-fold decreases in *survivin* gene and protein expression are shown in Figure 4C and Table 5, respectively. Using Western blotting, isomorellinol showed the highest potency in suppression of survivin protein expression (0.01 ×) (Table 5).

Western blotting analysis revealed that cells treated with isomorellinol resulted in a significant increase of AIF protein (Figure 5). Similar results were obtained for both CCA cell lines treated with the other three compounds (data not shown). Similar multi-fold increases in AIF protein expression were found for the four compound-treated cells as compared to controls (Table 5).

DISCUSSION

The present study demonstrated that the four caged xanthones; isomorellinol, forbesione, gambogic acid and isomorellin, exerted strong growth inhibition with different IC_{50} values in both CCA cell lines in a dose-dependent manner (Table 2 and Figure 2). The results indicate that these two CCA cell lines responded differently to different compounds. However, the potencies of these four compounds were very high, indicating their broad spec-

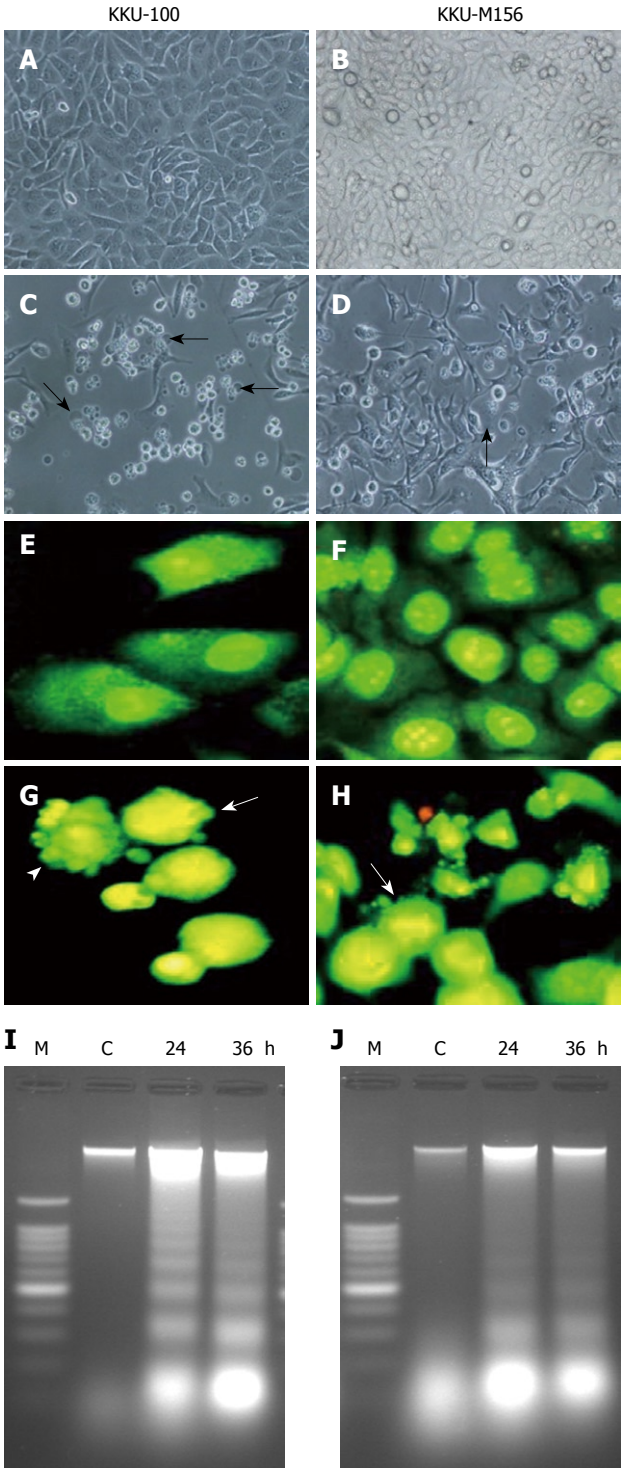


Figure 3 Induction of apoptosis in cholangiocarcinoma (CCA) cell lines by four caged xanthones. Photomicrographs (400 ×) of CCA cell lines KKU-100 (A, C) and KKU-M156 (B, D) treated with DMSO and gambogic acid for 48 h. A, B: Treated with DMSO; C, D: Treated with gambogic acid. Cells with membrane blebbing indicated by arrows; Fluorescence photomicrographs (400 ×) of CCA cell lines KKU-100 (E, G) and KKU-M156 (F, H) treated with DMSO and isomorellinol for 36 h followed by EB/AO staining. E, F: Treated with DMSO; G, H: Treated with isomorellinol. Nuclei of treated cells showing chromatin condensation (arrows) and nuclear fragmentation (arrow heads); DNA fragmentation of CCA cell lines treated with forbesione for the indicated times (24 h and 36 h). Genomic DNA was isolated and separated on 1.6% agarose gels containing 0.1 mg/mL ethidium bromide. I: KKU-100 cells; J: KKU-M156 cells. Lane C: DNA band of CCA cell lines treated with DMSO; Lane M: DNA markers. The figures show representative results of three independent experiments.

Table 3 Percentage of apoptotic cells in the four caged xanthone-treated and non treated CCA cell lines

Compounds	% apoptotic cells			
	KKU-100 (h)		KKU-M156 (h)	
	36	48	24	36
Control	8 ± 1.45	13 ± 0.66	8 ± 0.88	23 ± 0.66
Isomorellinol	44 ± 2.31[b]	79 ± 1.76[b]	34 ± 1.50[b]	89 ± 1.20[b]
Forbesione	34 ± 0.67[b]	78 ± 1.15[b]	38 ± 3.17[b]	83 ± 3.05[b]
Gambogic acid	32 ± 0.87[b]	70 ± 1.45[b]	17 ± 1.50[a]	72 ± 0.66[b]
Isomorellin	25 ± 1.21[b]	69 ± 1.76[b]	31 ± 0.58[b]	77 ± 4.16[b]

Data are mean ± SE of three independent experiments. [a]$P < 0.05$, [b]$P < 0.01$ *vs* control. CCA: Cholangiocarcinoma.

Table 4 Bax/Bcl-2 ratios of the four caged xanthone-treated and non treated CCA cell lines

Compounds	Bax/Bcl-2 ratio							
	KKU-100 (h)				KKU-M156 (h)			
	0	12	24	48	0	12	24	48
Isomorellinol	0.98	1.58	1.98	120	0.73	1.04	1.43	41.40
Forbesione	1.02	1.93	2.35	2.91	1.53	2.38	5.12	7.02
Gambogic acid	0.81	1.51	3.42	7.00	0.43	0.72	1.83	3.14
Isomorellin	0.98	1.98	3.12	5.20	0.56	0.70	1.00	1.90

Data are expressed as ratio of mean of protein expression level of Bax to Bcl-2 in control and the caged xanthone-treated CCA cell lines.

trum of anti-CCA effects. In addition, the selectivity of these compounds toward cancer cell lines compared to normal PBMCs was demonstrated (Table 2). Previously, these four caged xanthones have been shown to inhibit the growth of various mammalian cancer cell lines[10]. Gambogic acid was reported to be an effective telomerase inhibitor, displaying potent anticancer activity both *in vitro* and *in vivo*[8-11], and was shown to kill cancer cells selectively with no influence on normal cells[9]. The anticancer potencies of these caged xanthones were comparable to doxorubicin. Due to their potencies, the anticancer mechanisms of these four compounds deserve further investigation.

The growth inhibitory effect of these caged xanthones in both CCA cell lines was modulated through apoptosis which was evidenced by the typical morphological characteristics of apoptosis, such as cell shrinkage, membrane blebbing, chromatin condensation, nuclear fragmentation and DNA ladder formation (Figure 3). Cleavage of DNA at the inter-nucleosomal linker sites yielding DNA fragments has been used as a hallmark of apoptosis[20]. Consistent with our study, the apoptotic effects of gambogic acid in human breast tumor cells T47D[21] and human gastric carcinoma cells MGC-803[9] have been reported.

Apoptosis is regulated by several different genes in response to various stimuli. The Bcl-2 family of proteins plays major roles in apoptotic regulation through the mitochondrial pathway[22]. Our results show that these caged xanthones down-regulated the expression of the Bcl-2

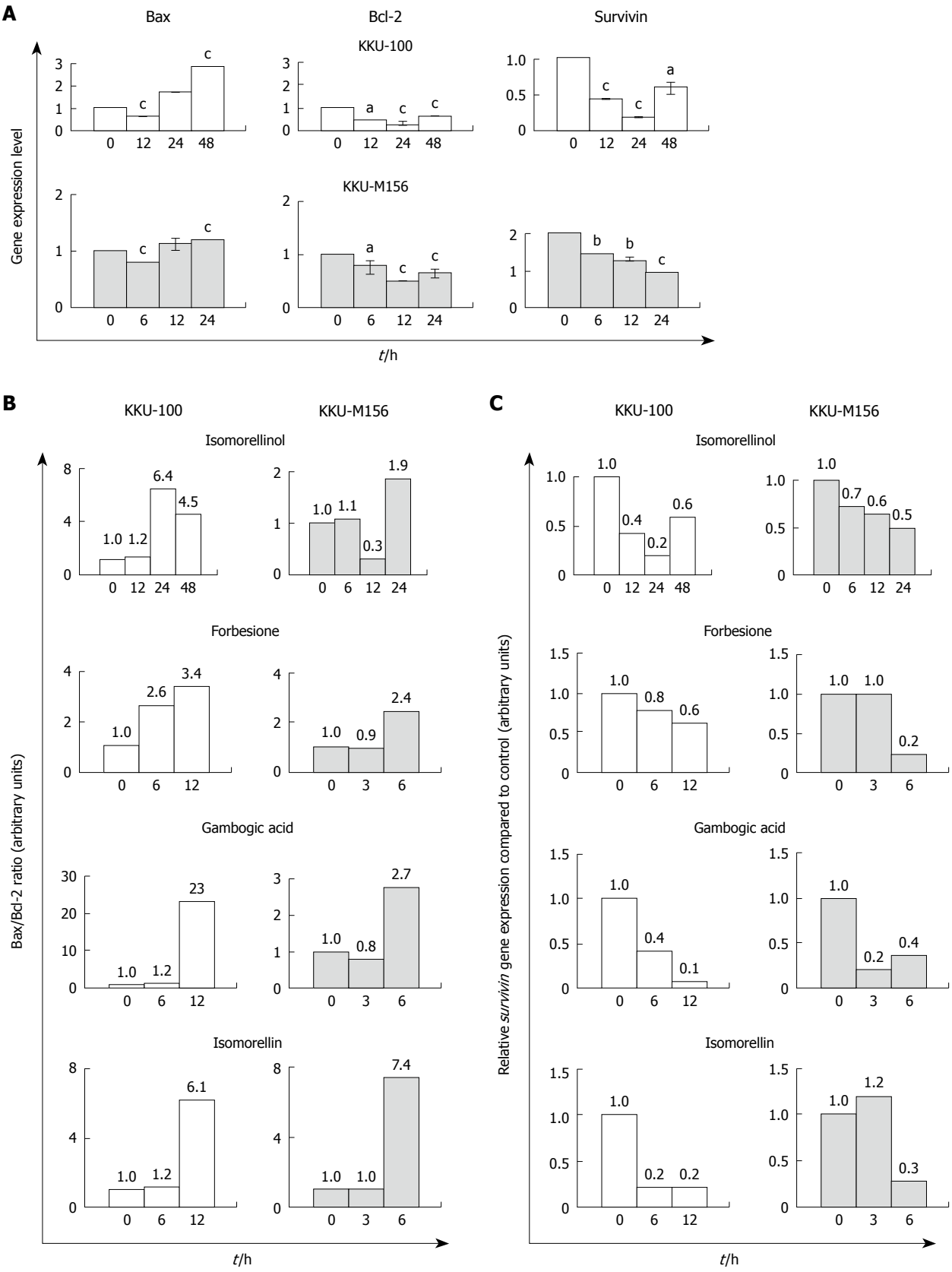


Figure 4 The four caged xanthones induce up-regulation of Bax with down-regulation of *Bcl-2* and *survivin* gene expressions in CCA cell lines. KKU-100 and KKU-M156 cells were treated with DMSO or the four caged xanthones for the indicated times. Real-time polymerase chain reaction for each gene was performed as described in Materials and Methods. Data were expressed as mean ± SE fold increase/decrease in mRNA expression compared to control, normalized with GAPDH (n = 3). $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ *vs* control. All four compounds showed the same result. A: The histograms of the isomorellinol-treated cells were used as a representative; B: Bax/Bcl-2 ratio; C: Relative *survivin* gene expression compared to control.

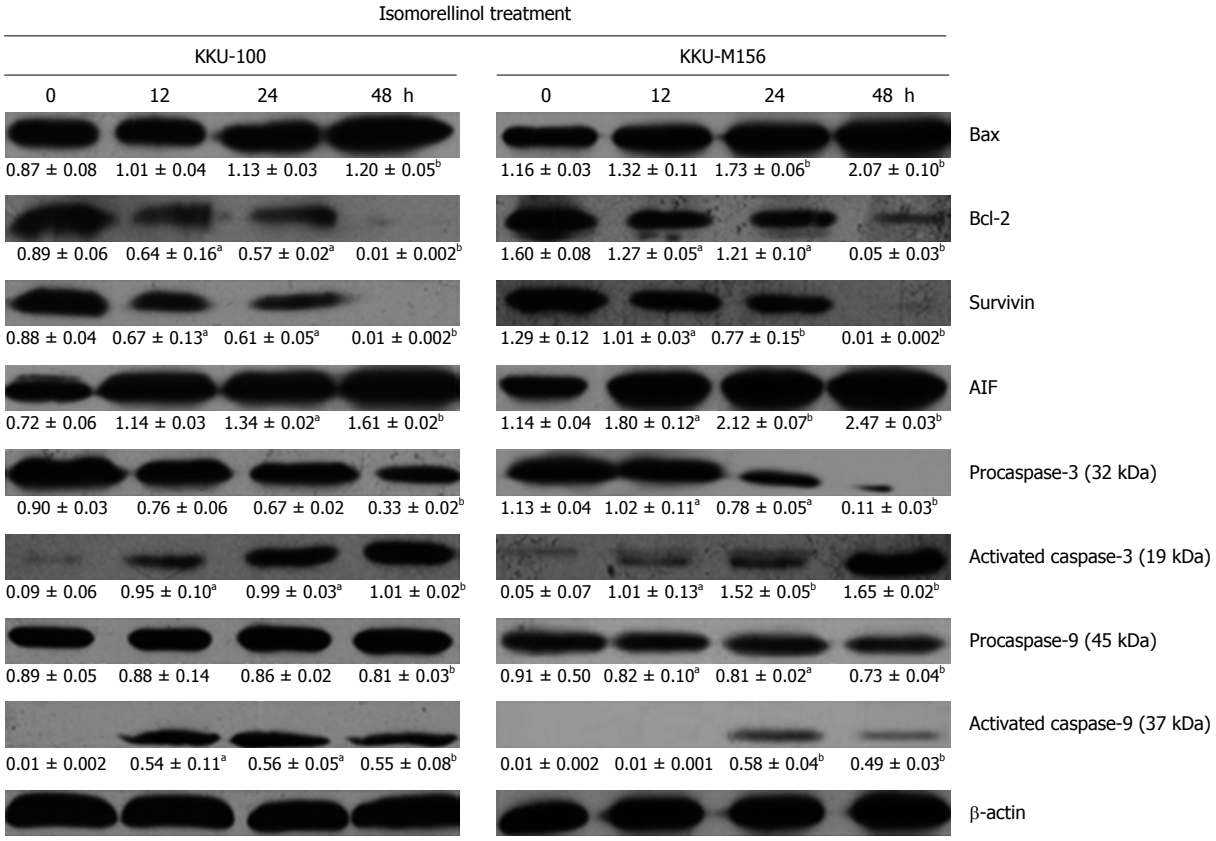


Figure 5 The four caged xanthones induce increase of Bax, AIF, activated caspase-3 and -9 while decreasing Bcl-2, survivin, procaspase-3 and -9 protein levels in CCA cell lines. KKU-100 and KKU-M156 cell lines were treated with DMSO or the four caged xanthones for the indicated times. Western blottings for each protein were performed as described in Materials and Methods. The mean ± SE of density reading of target protein normalized to β-actin was expressed in the respective box (n = 3). $^aP < 0.05$, $^bP < 0.01$ *vs* control. All four compounds showed the same result. The protein bands of the isomorellinol-treated cells were used as a representative.

Table 5 Fold decrease or increase of apoptotic-related proteins of the four caged xanthone-treated compared to non treated CCA cell lines

Apoptotic proteins	Compounds	Fold change in protein of the treated cells compared to control cells							
		KKU-100 (h)				KKU-M156 (h)			
		0	12	24	48	0	12	24	48
Activated caspase-9	Isomorellinol	1	54	56	55	1	1	58	49
	Forbesione	1	9.6	13.1	13	1	5.7	8	5.6
	Gambogic acid	1	30	78	60	1	2.6	4.5	3.9
	Isomorellin	1	6.3	12.3	9.3	1	1	29	34
Activated caspase-3	Isomorellinol	1	10.6	11	11.2	1	20.2	30.4	33
	Forbesione	1	6	9.6	12	1	5.7	9.6	10
	Gambogic acid	1	5.5	7.5	12.2	1	28	39.3	46.7
	Isomorellin	1	8.9	10	13	1	5	5.7	12
Survivin	Isomorellinol	1	0.8	0.7	0.01	1	0.8	0.6	0.01
	Forbesione	1	0.5	0.54	0.3	1	0.7	0.54	0.5
	Gambogic acid	1	0.6	0.4	0.3	1	0.7	0.6	0.4
	Isomorellin	1	0.6	0.5	0.3	1	0.8	0.5	0.3
AIF	Isomorellinol	1	1.6	1.9	2.3	1	1.6	1.9	2.2
	Forbesione	1	1.2	1.6	2.1	1	1.6	2.8	4
	Gambogic acid	1	1.5	1.6	2.7	1	1.6	2.1	3.4
	Isomorellin	1	1.9	3.3	3.5	1	1.3	1.7	2.5

Data are expressed as ratio of mean of protein expression level of the apoptotic-related proteins in the caged xanthone-treated to non treated CCA cell lines.

gene and protein while up-regulating the expression of Bax gene and protein, leading to an increase in the Bax/Bcl-2 ratio (Figure 4B and Table 4). In agreement with our study, gambogic acid-induced apoptosis by regulation of the expression of Bax and Bcl-2 proteins in the human gastric carcinoma cell line MGC-803 has been reported[9]. Bax protein has previously been reported to induce the intrinsic apoptosis pathway by inhibiting the function of

Bcl-2[23], and by increasing the release of death-mediated proteins such as cytochrome C, Smac/DIABLO and AIF from the inter-membrane space to the cytosol[24,25]. Our results show a significant increase in the level of AIF protein in response to the four caged xanthone treatments (Figure 5 and Table 5). This result suggests that the AIF, caspase-independent mitochondrial pathway[25] may be involved in apoptosis induction in the caged xanthone-treated cells.

In the intrinsic apoptotic pathway, the release of cytochrome C from mitochondria triggers caspase-9 and -3 activation through formation of the apoptosome complex. Then the activated caspase-3 is able to cleave multiple cellular substrates leading to DNA fragmentation[24]. Our results show that caspase-9 and -3 were activated by the caged xanthones (Figure 5 and Table 5), suggesting that these caged xanthones induced apoptosis in both CCA cell lines through caspase-9 and -3 activation. Previously, mitochondrial destabilization and caspase-3 activation were reportedly involved in the apoptosis of Jurkat cells induced by gaudichaudione A, a cytotoxic xanthone[26]. Gambogic acid reportedly induced apoptosis and activated caspases in T47D cells[21].

The family of human inhibitor of apoptosis proteins (IAP), including XIAP, c-IAP1, c-IAP2, NAIP and survivin, have been reported to be direct inhibitors of activated caspase-3 and -7[27,28]. Our data show that *survivin* gene and protein expressions were decreased by the four caged xanthones (Figure 4A and C, and Table 5). Since these compounds were found to induce apoptosis in both CCA cell lines by activation of caspase-3, the factor contributing to caspase-3 activation in these treated cells may be a decreased survivin expression. Previously, gambogic acid has been reported to down-regulate IAP, IAP1 and IAP2 in human myeloid leukemia cells[29].

Using Western blotting, isomorellinol was found to have the highest potency in increasing the Bax/Bcl-2 ratio (Table 4) and in decreasing survivin protein expression (Figure 5 and Table 5). This result was also confirmed by the greatly increased amount of activated caspase-3 and -9 in both isomorellinol-treated CCA cell lines (Table 5). Gambogic acid was shown to have lower potency in increasing the Bax/Bcl-2 ratio (Table 4) and decreasing survivin protein expression (Table 5); whereas the increased level of activated caspase-3 was similar to that of isomorellinol (Table 5). These results suggest that the activation of caspase-3 might be stimulated by caspase-12 through endoplasmic reticulum stress-induced apoptosis[30]. Comparing these two CCA cell lines, both isomorellinol and gambogic acid showed higher potency in increasing activated caspase-3 in KKU-M156 cells than KKU-100 cells (Table 5). This may be due to the different histological type of the CCA cell lines. KKU-100 is a poorly differentiated adenocarcinoma which has a higher resistance to chemotherapeutic drugs than KKU-M156, a moderately differentiated adenocarcinoma[13]; whereas forbesione and isomorellin showed the same potency against both CCA cell lines. The latter result may be due to the different functional groups present in these four caged xanthones.

The chemical structure diversity of the four compounds reflects the biological activities. The common feature of all four compounds is the presence of the novel caged structure, which is believed to be a prerequisite for potent anticancer activity in comparison with normal xanthones and chromyl xanthones. The chromene ring is absent in forbesione compared with the other three compounds. Forbesione has only two non-functionalized prenyl side-chains, whereas isomorellin, isomorellinol and gambogic acid are highly prenylated with one of the prenyl methyl groups functionalized as an aldehyde, a primary alcohol and a carboxylic acid functional group, respectively. The functional group variations do not appear to affect the anticancer activity against the two CCA cell lines; however, at a mechanistic level, isomorellinol exhibited the highest potency in increasing the Bax/Bcl-2 ratio and in decreasing survivin protein expression. Other activities at the molecular level indicate that functional groups on the prenyl side chain may be important. It is premature to conclude what the definite structure-activity relationship may be but this preliminary insight provides a basis for further medicinal chemistry studies.

In conclusion, our findings demonstrate for the first time that the four caged xanthones, isolated from *G. hanburyi,* are capable of inhibiting proliferation and inducing apoptosis in CCA cell lines. This is also the first report which shows the molecular mechanism of the growth inhibitory effects of isomorellinol, forbesione and isomorellin. These compounds induce down-regulation of Bcl-2 protein while up-regulating the Bax and AIF proteins, leading to the activation of caspase-9 and -3 and DNA fragmentation. Moreover, the down-regulation of survivin protein thus maintains caspase-3 in an active state and stimulates the molecular cascade of apoptosis. Based on these results, we conclude that these four caged xanthones stimulate both caspase- and non-caspase mitochondrial-dependent apoptosis in KKU-100 and KKU-M156 cells. Furthermore, it appears the mitochondrial apoptosis pathway is induced by the upstream receptor-ligand mediated apoptosis pathway. The precise target of action of these four caged xanthones therefore remains to be elucidated. There is a potential role for these caged xanthones in cancer therapy.

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COMMENTS

Background

Cholangiocarcinoma (CCA) is a malignant tumor, characterized by a poor prognosis and unresponsive to conventional chemotherapeutic agents. Therefore, searching for novel and effective therapeutic agents for CCA is necessary. Several caged xanthones from *Garcinia hanburyi* (*G. hanburyi*) have been reported to be potent antiproliferatives, as well as having anticancer and anti-tumor activities, and induce apoptosis in various cancer cell lines.

Research frontiers

The four caged xanthones from *G. hanburyi*; isomorellin, isomorellinol, forbesione and gambogic acid, exerted strong growth inhibition in both KKU-100 and KKU-M156 cells, indicating their broad spectrum of anti-CCA effects. These compounds were shown to kill cancer cells selectively with no influence on normal peripheral blood mononuclear cells (PBMCs). Growth suppression by these compounds was due to apoptosis which correlated with an increase in Bax/Bcl-2 ratio and apoptosis-inducing factor, activation of caspase-9 and -3, and a decrease in survivin expression. At mechanistic level, isomorellinol exhibited the highest potency.

Innovations and breakthroughs

This is the first report which shows the growth inhibitory effect and molecular mechanisms of the four caged xanthones isolated from *G. hanburyi* in CCA cell lines. The results suggest that these compounds stimulate both a caspase- and non-caspase mitochondrial-dependent signaling pathway in CCA cell lines.

Applications

The chemical structure diversity of the four compounds reflects the biological activities. At the molecular level, isomorellinol exhibited the highest potency, indicating that functional groups on the prenyl side chain may be important. It is premature to conclude what the definite structure-activity relationship may be, but this preliminary insight provides a basis for further medicinal chemistry studies. Based on these results, The authors suggest that these four caged xanthones are compounds with great promise and may serve as a potential source and lead-structure for the development of a drug for the treatment of CCA.

Peer review

The manuscript by Hahnvajanawong *et al* evaluates the mechanism by which various caged xanthones exert their antiproliferative activity on cholangiocarcinoma cell lines. The data presented are convincing and the manuscript well written.

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ORIGINAL ARTICLE

Roux-en-Y gastric bypass promotes expression of PDX-1 and regeneration of β-cells in Goto-Kakizaki rats

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Abstract

AIM: To study the effects of Roux-en-Y gastric bypass (RYGB) on the expression of pancreatic duodenal homeobox-1 (PDX-1) and pancreatic β-cell regeneration/neogenesis, and their possible mechanisms in diabetics.

METHODS: Three groups of randomly selected non-obese diabetic Goto-Kakizaki (GK) rats were subjected to RYGB, sham-RYGB and sham-operation (sham-op) surgery, respectively. The rats were euthanized at post-operative 1, 2, 4 and 12 wk. Their pancreases were resected and analyzed using reverse transcription polymerase chain reaction to detect the mRNA of PDX-1. Anti-PDX-1 immunohistochemical (IHC) staining and Western blotting were used to detect the protein of PDX-1. Double IHC staining of anti-Brdu and -insulin was performed to detect regenerated β-cells. The index of double Brdu and insulin positive cells was calculated.

RESULTS: In comparison with sham-RYGB and sham-op groups, a significant increase in the expressions of PDX-1 mRNA in RYGB group was observed at all experimental time points (1 wk: 0.378 ± 0.013 *vs* 0.120 ± 0.010, 0.100 ± 0.010, $F = 727.717$, $P < 0.001$; 2 wk: 0.318 ± 0.013 *vs* 0.110 ± 0.010, 0.143 ± 0.015, $F = 301.509$, $P < 0.001$; 4 wk: 0.172 ± 0.011 *vs* 0.107 ± 0.012, 0.090 ± 0.010, $F = 64.297$, $P < 0.001$; 12 wk: 0.140 ± 0.007 *vs* 0.120 ± 0.010, 0.097 ± 0.015, $F = 16.392$, $P < 0.001$); PDX-1 protein in RYGB group was also increased significantly (1 wk: 0.61 ± 0.01 *vs* 0.21 ± 0.01, 0.15 ± 0.01, $F = 3031.127$, $P < 0.001$; 2 wk: 0.55 ± 0.00 *vs* 0.15 ± 0.01, 0.17 ± 0.01, $F = 3426.455$, $P < 0.001$; 4 wk: 0.39 ± 0.01 *vs* 0.18 ± 0.01, 0.22 ± 0.01, $F = 882.909$, $P < 0.001$; 12 wk: 0.41 ± 0.01 *vs* 0.20 ± 0.01, 0.18 ± 0.01, $F = 515.833$, $P < 0.001$). PDX-1 mRNA and PDX-1 protein production showed no statistical significance between the two sham groups. Many PDX-1 positive cells could be found in the pancreatic islets of the rats in RYGB group at all time points. In addition, the percentage of Brdu-insulin double staining positive cells was higher in RYGB group than in the other two groups (1 wk: 0.22 ± 0.13 *vs* 0.03 ± 0.06, 0.03 ± 0.06, $P < 0.05$; 2 wk: 0.28 ± 0.08 *vs* 0.00 ± 0.00, 0.03 ± 0.06, $P < 0.05$; 4 wk: 0.24 ± 0.11 *vs* 0.07 ± 0.06, 0.00 ± 0.00, $P < 0.001$; 12 wk: 0.20 ± 0.07 *vs* 0.03 ± 0.06, 0.00 ± 0.00, $P < 0.05$).

CONCLUSION: RYGB can increase the expression of pancreatic PDX-1 and induce the regeneration of β-cells in GK rats. The associated regeneration of islet cells may be a possible mechanism that how RYGB could improve type 2 diabetes mellitus.

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Key words: Gastric bypass; Diabetes mellitus; Regene-

ration; β-Cells; Animals; Pancreatic duodenal homeobox-1

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INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disease characterized by insulin resistance and progressive deterioration of islet functions[1]. The apoptosis of β-cells may be a major contributor to the failure of these cells at the late stages of T2DM[2]. An effective treatment for diabetes can improve the functions of β-cells and increase their numbers. Roux-en-Y gastric bypass (RYGB) is a commonly used surgical treatment for patients with morbid obesity. It could not only significantly and persistently reduce patients' body weight, but also lower the serum levels of glucose, and glycosylated hemoglobin in 80%-100% of T2DM patients[2,3]. Furthermore, this treatment could also prevent impaired glucose intolerance from developing into diabetes among these patients[3]. Animal experiments have shown that RYGB treatment can improve the functions of pancreatic islets and the metabolism of glucose in both obese and non-obese diabetic rats[4]. These results suggest that RYGB surgery is effective in the treatment of diabetes.

Efforts have been made to understand the mechanism of RYGB treatment on T2DM patients, however, the exact mechanism still remains elusive[5,6]. RYGB treatment may be correlated to the changes of gastrointestinal hormones, which then influence the enteroinsular axis to regulate the endocrine function of the pancreas[7]. One of the most important intestinal hormones involved in this mechanism is glucagon-like peptide 1 (GLP-1). It has been found that RYGB surgery can increase GLP-1 levels in the sera of T2DM patients and in the experimental animals. This effect may last 1 year and in some cases, even up to 20 years after RYGB surgery[5,8]. This may be one of the reasons why the post-operative diabetes is well controlled in both human beings and experimental rodents.

Recently, it has also been reported that after RYGB surgery, some patients developed nesidioblastosis, a serious life-threatening postprandial hyperinsulinism with hypoglycemia[9-12]. These symptoms may be alleviated, after part of the pancreas is removed. Hypertrophy and hyperplasia pancreatic islets are common features seen in the resected specimens, and even multiple islet tumors detected in some specimens. Since the hyperplasia of islet cells are rarely found in the normal population[9,12,13], the above data suggest that RYGB may be associated with the regeneration and neogenesis of the pancreatic islets. Thus, RYGB surgery may occasionally cause the pathological overgrowth and hyperplasia of β-cells in the islets[14]. However, this hypothesis has not been supported by any animal experiments.

Pancreatic duodenal homeobox-1 (PDX-1) is known as the first and most important transcription factor expressed during the embryonic development of the pancreas in experimental animals. All pancreatic cells are derived from the precursor cells with expression of PDX-1[15]. The embryonic pancreas failed to develop in the PDX-1 knockout mice[16]. The mice with heterozygous mutations of *PDX-1* gene could develop a normal pancreas, but may show abnormal glucose tolerance and diabetes in adulthood[17]. If PDX-1 is removed from mature β-cells by Cre-recombinase-mediated deletion, the expression of these genes is impaired and the loss of β-cells is accelerated[18]. The expression level of PDX-1 in the pancreas reach the peak in the first 10.5 d during the development of mouse embryos, and decline gradually afterwards. After the birth and in the adulthood, the expression of PDX-1 becomes very weak, which is specifically found only in 90% of β-cells and 10% of δ-cells in the islets[19].

PDX-1 is significantly re-expressed in proliferating cells in the ducts[20,21] and islets[22] during pancreas regeneration[23] and may serve as a marker of cells that regain their pluripotency to differentiate into all types of pancreatic cells. Subtotal pancreatectomy promoted the PDX-1 expression of duct epithelial cells in Sprague-Dawley rats[21], and the PDX-1 positive cells were also obvious in mouse model with pancreas injury[24]. PDX-1 has significant effect on cell proliferation and could induce a dramatic cell proliferation, which is similar to the formation of the pancreas during embryonic development[25]. Studies of animal models suggested that the down-regulation of PDX-1 expression in the β-cells may underlie the pathogenesis of β-cell failure and the onset of T2DM[26]. Therefore, the regeneration of islet β-cells requires the activation and expression of PDX-1. PDX-1 expression is the basis of regeneration of the pancreas.

Goto-Kakizaki (GK) rats are used in a genetically non-overweight type 2 diabetes model, which has the features of slightly elevated blood glucose, impaired secretion of glucose-stimulated insulin and reduced mass of pancreatic β-cells[1]. It is similar to those in T2DM patients. Therefore, GK rats have been widely used in an experimental animal model for T2DM studies. The aim of this study was to investigate the effects of RYGB surgery on the expression of PDX-1 as well as on the regeneration of pancreatic β-cells in non-obese diabetic GK rats, and the possible mechanisms.

MATERIALS AND METHODS

Animals

Sixty male GK rats weighing 200-230 g, at age of 10-12 wk, were provided by Shanghai Laboratory Animal Center,

Chinese Academy of Sciences, and kept in the animal facility at the Henan Key Lab of Biological Psychiatry, China. The rats were randomly divided into 3 groups with 20 rats in each group: RYGB group, sham-RYGB group and sham-operation (sham-op) group.

Abdominal surgeries

For experiments, GK rats in the RYGB group were fasted overnight and anesthetized with 4% chloral hydrate (Wako Co., Japan). Upper abdomen was cut open with midline incision and then the stomach was sutured bisecting it to produce two separate gastric cavities, distal one and a proximal one. The jejunum was cut off about 8 cm from the Tretiz ligaments. The proximal part of the stomach was opened and anastomosed with the distal jejunum. The proximal jejunum was anastomosed with the side of distal jejunum at the site about 10 cm away from the above stomach-jejunum anastomosis. The abdominal incisions were closed with absorbable sutures. For controls, the stomachs of rats were transected across the pylorus followed by discontinuously suturing back the two gastric stumps in the sham-RYGB group, and in the sham-op group there was only an anterior abdominal wall incision which was immediately sutured without disturbing the internal organs. After surgery, all rats were fasted and only fed with water on the same day of the operation (day 0). They were fed with food starting from day one. Animals were administered with the antibiotics, cefradine powder (Bright Future Pharmaceuticals Factory, Hong Kong), at a dose of 100-150 mg/kg body weight, once every 24 h, for a total of 3 d to prevent infections. Animals were euthanized on the 1st, 2nd, 4th and 12th wk after operation, with five rats from each group at each time-point, separately.

Reverse transcription polymerase chain reaction

On the sacrifice day, each rat was first intraperitoneally injected with 5-bromo-2-deoxyuridine (Brdu, from Sigma, USA) at 50 mg/kg and two hours later, the rats were euthanized with overdose anesthesia. The pancreatic tissues were isolated and rinsed with 0.01 mol/L phosphate buffered saline (PBS). Eighty mg of pancreatic tissues from each rat was homogenized using Trizol reagent (Sigma, USA), and the total RNA was extracted according to the manufacturers' protocol. The optical density of RNA was read with the spectrophotometry at the wavelength of 260 nm (A_{260}). The cDNAs were synthesized by adding 1-2 μg DNA-free RNA to the RT mixture. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as an internal control. PCR was performed with the following primers: GAPDH (forward: 5'-ACCACAGTCCATGCCATCAC-3'; reverse: 5'-TCCACCACCCTGTTGCTGTA-3'), PDX-1 (forward: 5'-CCAAAACCGTCGCATGAAGTG-3', reverse: 5'-TCTGGGTCCCAGACCCG-3'). The combination of the DNA primers produced single PCR products of 550 and 208 bp in length, respectively. PCR was carried out in a DNA thermal cycler (Biometra, Goettingen, Germany) after denaturation at 95℃ for 5 min. The number of total PCR cycles was 35 for PDX-1 and 30 for GAPDH, and each cycle consisted of denaturation at 94℃ for 15 s, annealing at 65℃ (GAPDH at 62℃) for 30 s, and extension at 72℃ for 90 s. For semiquantitative analysis, 25 μL of each PCR product was examined by electrophoresis in 1.8% agarose gel containing 0.5 μg/mL of ethidium bromide. DM 1000 (LEICA, Germany) was used as a molecular marker. OD readings of PDX-1 mRNA were normalized to those of GAPDH mRNA, and the ratios were analyzed.

Paraffin sectioning

The pancreatic tissues obtained from the above rats were also immediately fixed with 4% paraformaldehyde at 4℃ overnight. The tissues were then dehydrated in graded ethanol, cleared in xylene and finally embedded in paraffin. The tissues were sectioned to produce 4 μm-thick sections, which were mounted onto slides for further staining.

Immunohistochemical staining

In brief, the pancreatic sections were deparaffined in xylene and rehydrated in graded ethanol. The sections were blocked in H_2O_2 and sequentially incubated with goat anti-PDX-1 polyclonal primary antibody (1:50 dilution; Santa Cruz, USA) overnight and anti-goat secondary antibody (1:100 dilution; Santa Cruz, USA) for 2 h. For negative control, the primary antibody was replaced with 0.01 mol/L PBS.

Western blotting

Total protein was obtained from the pancreatic tissues, that were lysed in the buffer containing 50 mmol/L Tris, pH 7.5, 0.3 mol/L NaCl, 0.5% Triton X-100 and 0.1% sodium azide, and protease inhibitor cocktail CLAP (chymostatin, leupeptin, aprotinin and pepstatin, 100 μmol of each, Sigma Aldrich, USA). Then protein samples (50 μg) were separated in 10% sodium dodecyl sulphate polyamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (Amersham, UK). The membranes were then blocked in 0.01 mol/L PBS containing 5% dry milk and 0.3% Tween 20 for 1 h. The membranes were then incubated with goat anti-PDX-1 antibody (1:10000 dilution) at room temperature for 2 h and horseradish peroxidase-labelled secondary antibody (1:5000 dilution; Amersham, Japan) at room temperature for 1 h, respectively. Finally, the proteins were visualized on enhanced chemiluminescence hyperfilm (Amersham, Japan) by application of Amershem's Enhanced Chemiluminescence Western blotting Detection System. The band intensity was quantitated with GelAnalysis software (GreyStone-Iconix).

Double immunohistochemical staining

Briefly, the pancreatic sections were sequentially incubated with guinea pig anti-insulin polyclonal antibody (1:50 dilution; Santa Cruz, USA), biotinylated rabbit anti-guinea pig antibody (1:50 dilution; Santa Cruz, USA), and streptavidin-alkaline phosphatase complex (Santa Cruz, USA), for a period of 45 min each. The alkaline phosphatase activity was identified using new fuchsin under light microscope. The sections were counterstained with Harris hematoxylin.

To detect the second antigen Brdu, the sections were placed in 2 N hydrochloric acid and kept at 70℃ for 15 min for antigen retrieval. The reaction was stopped by 0.1 mol/L sodium borate and left in 0.01 mol/L PBS for 20 min. Tissue sections were incubated with hydrogen peroxide for 45 min, mouse anti-Brdu monoclonal antibody for 3 h, biotinylated goat anti-mouse antibody for 45 min, and streptavidin-peroxidase complex for 45 min, respectively. Finally, the peroxidase activity was visualized with diaminobenzidine under light microscope, and the reaction was stopped by rinsing with PBS. Avidin and biotin were then applied to the sections for 45 min each.

Cell count

The images of the above double-stained sections were captured at 100 × magnifications. In each rat, a total of 1000 nuclei from five sections were counted, and the sections were randomly selected from tail (3 sections), body (1 section) and head (1 section). The index of Brdu-labeling was calculated as follows: The index of Brdu labeling = (The number of double-stained cells/1000) × 100%.

Statistical analysis

All data were presented as mean ± SD (n = 5). The data were analyzed using SPSS13.0 statistical package. The differences were analyzed by factorial design of variance. The comparison of variance between two groups was analyzed by LSD method. Missing variance was analyzed using Dunnett T3. Non-normal data were analyzed non-parametrically using a number of independent samples and tested by Kruskal-Wallis method. $P < 0.05$ was considered to indicate statistical significance of the test results.

RESULTS

Reverse transcription polymerase chain reaction

Compared with internal control, GAPDH, the data of relative mRNA expression of PDX-1 at weeks 1, 2, 4 and 12 after surgery were 0.378 ± 0.013, 0.318 ± 0.013, 0.172 ± 0.011 and 0.140 ± 0.007 in the rat pancreas of RYGB group, 0.120 ± 0.010, 0.110 ± 0.010, 0.107 ± 0.012 and 0.120 ± 0.010 in sham-RYGB group, and 0.100 ± 0.010, 0.110 ± 0.010, 0.090 ± 0010 and 0.097 ± 0.015 in sham-op group, respectively. The above data were plotted as shown in Figure 1B. The F values of the relative expression level of PDX-1 mRNA in the rat pancreas at 1, 2, 4 and 12 wk after surgery in the three groups were 727.717, 301.509, 64.297 and 16.392, respectively, all $P < 0.05$. There was statistically significance at each time point. In addition, the relative mRNA expression level of PDX-1 in the RYGB group showed statistical significance (F = 512.105, $P < 0.05$), being higher at 1 and 2 wk after surgery. In contrast, there was no significant difference at different time points between sham-RYGB and sham-op groups (F = 0.750, both $P > 0.05$).

Immunohistochemical staining

Anti-PDX-1 immunohistochemical (IHC) staining showed that there were many PDX-1 positive cells with brown nuclei in the rat pancreas in the RYGB group. These cells were mainly located in the islets. A few PDX-1 positive cells could be observed in the ducts and acini (Figure 2A and B). In contrast, few PDX-1 positive cells were found in the islets of rat pancreas at each time point in both sham-RYGB and sham-op groups (Figure 2C).

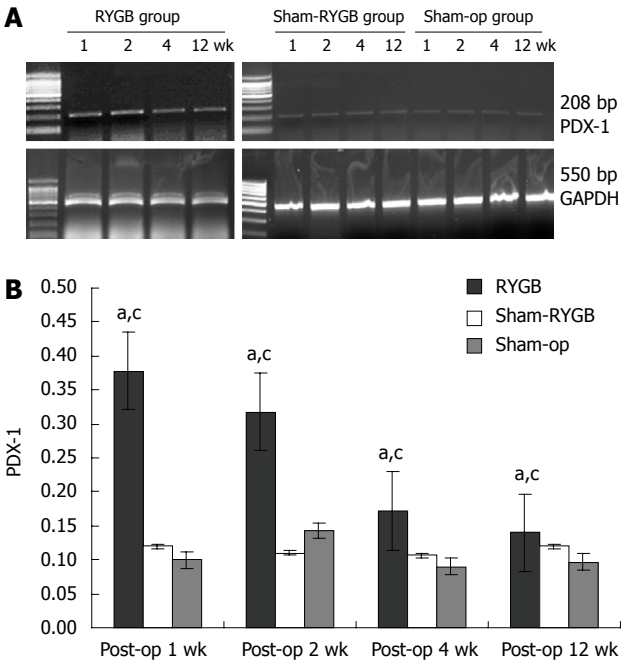


Figure 1 The expression of PDX-1 mRNA in the pancreas of GK rats. A: Representative gel from RT-PCR with primers to PDX-1 (208 bp) and GAPDH (550 bp); B: The intensities of PDX-1 mRNA bands relative to those of GAPDH bands. $^{a}P < 0.05$ *vs* sham-RYGB; $^{c}P < 0.05$ *vs* sham-op group.

Western blotting

The data of the expressions of PDX-1 protein in the rat pancreas are plotted in Figure 3A. Compared with the internal control, GAPDH, the relative expressions of PDX-1 protein in the rat pancreas are shown in Figure 3B. The F values of the relative expression of PDX-1 protein in the rat pancreas at 1, 2, 4 and 12 wk after surgery in the three groups were 3031.127, 3426.455, 882.909 and 515.833, respectively (all $P < 0.05$). In addition, there was statistical significance at each time point among the three groups. The relative expression levels of PDX-1 protein in the rat pancreas of RYGB group showed statistical significance (F = 676.157, $P < 0.05$), being highest at 1 and 2 wk after surgery.

Double IHC staining

Anti-Brdu and anti-insulin double stained cells could be found in the rat pancreas in the RYGB group at each time point (Figure 4A-C). The Brdu and insulin double positive cells were found primarily in the islets and occasionally in the ducts (Figure 4C). Single insulin positive cells could be observed in the acini (Figure 4B) and there were many single Brdu positive cells in the acini of the pancreas (Figure 4A-D). However, few Brdu and insulin double positive cells could be found in the rat pancreas in both sham-RYGB

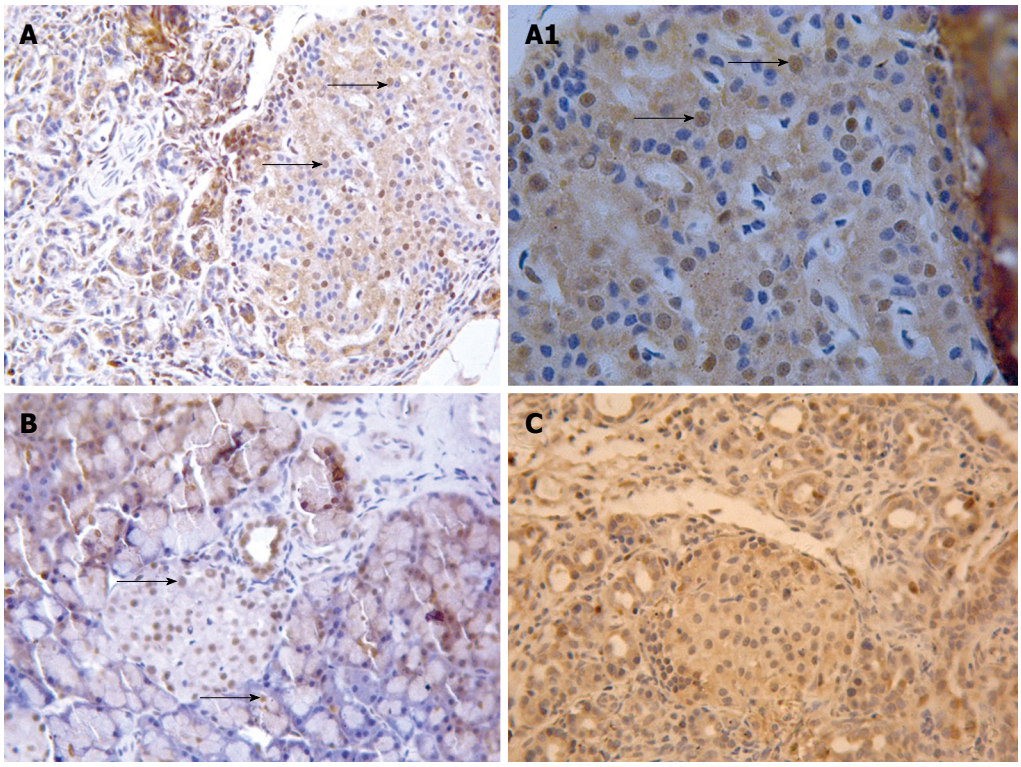


Figure 2 The expression of PDX-1 protein in the pancreas of GK rats. PDX-1 positive signals were stained in dark brown color (arrows), and mainly located the islets. Occasionally, they were found in the duct and acinar cells. A, B and A1 in RYGB group; C in sham-op group. (Hematoxylin & anti-PDX-1 IHC stain. A, B and C at 200 × magnifications; A1, at 400 × magnifications).

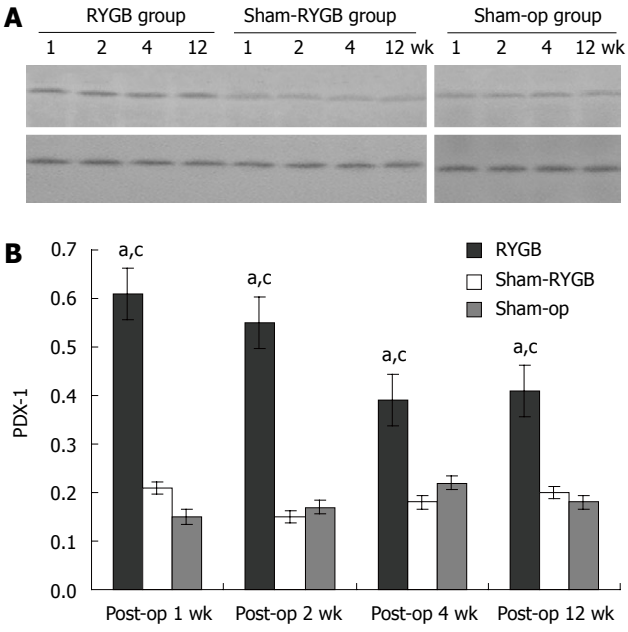


Figure 3 Western blotting analysis of PDX-1. A: Representative Western blotting analysis of PDX-1 protein; B: The relative expression level of PDX-1 protein. $^{a}P < 0.05$ *vs* sham-RYGB; $^{c}P < 0.05$ *vs* sham-op group.

and sham-op groups at each time point after surgery. The percentage of both Brdu and insulin positive cells showed a significant increase at 1, 2, 4 and 12 wk after surgery as compared with the other two groups (Figure 4E). The χ^2 values were 6.197, 8.485, 7.808 and 8.166, respectively; the corresponding *P* values were 0.045, 0.014, 0.020 and 0.017. The rank of RYGB group was the highest at each time point. In addition, there were more single Brdu positive cells in RYGB group than in the other two groups.

DISCUSSION

In this study, the expression levels of PDX-1 mRNA in the RYGB group were significantly higher than those in the sham-RYGB and sham-op groups at all time points of 1, 2, 4 and 12 wk after surgery. It suggests that RYGB surgery may result in an increase in the expression of pancreatic PDX-1 mRNA in GK rats. In other words, RYGB surgery may promote the expression of PDX-1 mRNA, which may last at least 12 wk. The expression of PDX-1 mRNA in RYGB group peaked at the first two weeks and decreased afterwards, which may suggest that RYGB surgery played a role in promoting the expression of PDX-1 mRNA primarily at the early stage after the surgery.

Similarly, the protein expressions of PDX-1 in RYGB group were also significantly higher than those in the other two groups at all time points. The protein expression level of pancreatic PDX-1 in RYGB group also peaked at the first two weeks after surgery, and decreased afterward. Furthermore, the results of anti-PDX-1 IHC staining also showed that PDX-1 positive cells in the pancreas of GK rats increased significantly in RYGB group when compared with those in both sham-RYGB and sham-op groups. The PDX-1 positive cells were mainly located in the islets, but not in the acinus and ducts.

It is important to increase the number of β-cells in the pancreatic islets in the treatment of diabetes. The regenerated β-cells in the islets may be double labeled by anti-Brdu and anti-insulin double IHC staining. In this experiment, Brdu and insulin double positive cells were visible in the pancreas in all GK rats at all time points. In addition, the percentages of Brdu and insulin double positive cells in RYGB group were significantly higher than those in both sham-RYGB and sham-op groups. The results also suggest

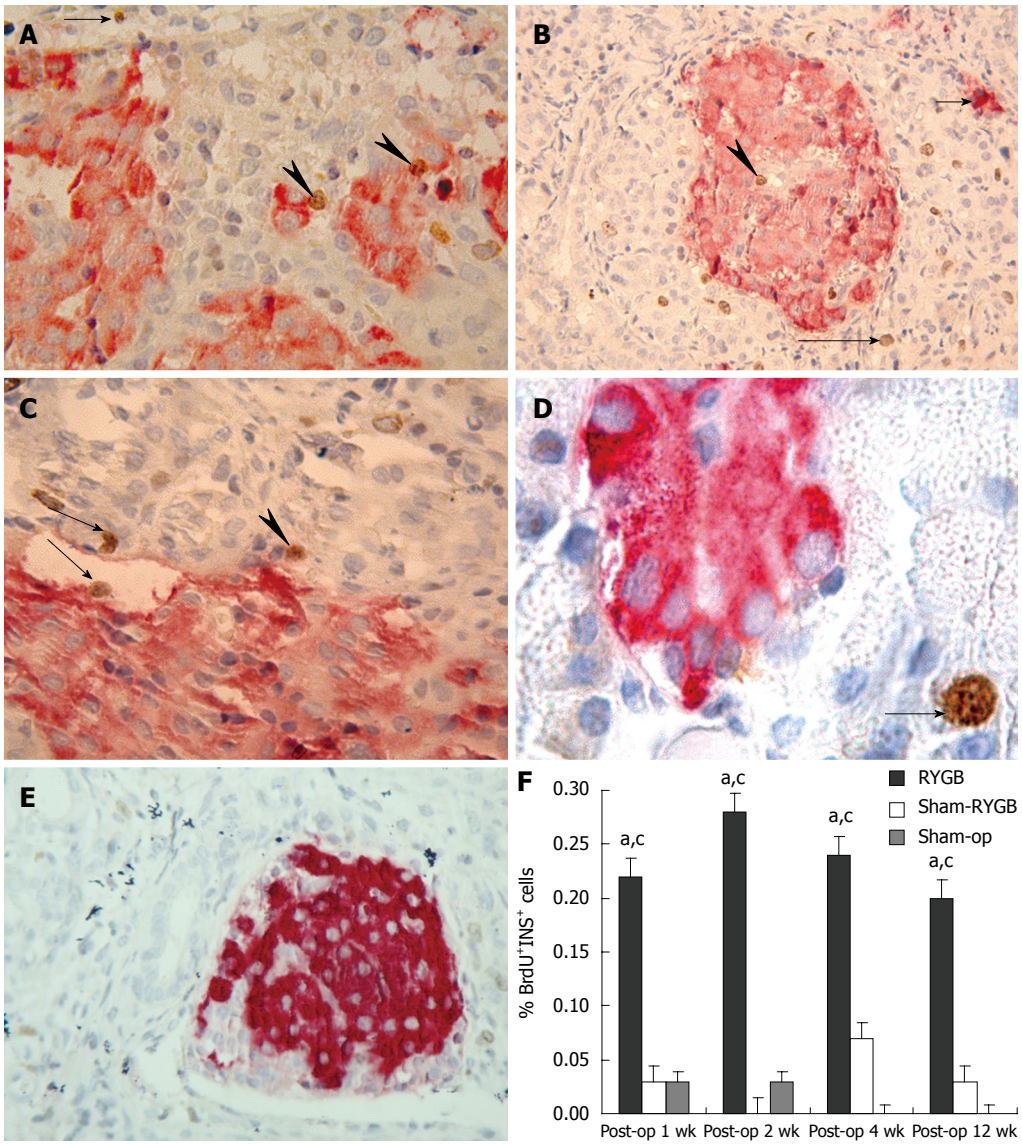


Figure 4 Anti-Brdu and -insulin double IHC staining. A: Brdu and insulin double positive cells in the islets or acini (arrowheads), single Brdu positive cells in the acini (thin-tail arrow); B: Brdu and insulin double positive cells in the islets (arrowhead) and single Brdu positive cells in the acini (thin-tail arrow), insulin positive cells in the acini (short-tail arrow); C: Brdu and insulin double positive cells in the ducts (thin-tail arrows) and islets (arrowhead); D: Single Brdu positive cells in the acini (thin-tail arrow); E: No Brdu and insulin double labeling cells in the pancreas of sham-RYGB rats; F: The index of Brdu and insulin double positive cells. (A and C at 400 × magnifications; B and E at 200 × magnifications; D at 1000 × magnifications). $^{a}P < 0.05$ *vs* sham-RYGB; $^{c}P < 0.05$ *vs* sham-op group.

that RYGB surgery may promote the regeneration of islet β-cells in GK rats, which is likely another mechanism why RYGB surgery may decrease the blood glucose in diabetes patients.

The results in this study have shown that RYGB surgery may result in an increase in expressions of PDX-1 mRNA and PDX-1 protein, the number of PDX-1 positive cells and the percentage of Brdu and insulin double positive cells in GK rats. All these suggest a regeneration of islet β-cells, which may be related to persistent elevations of serum GLP-1 after RYGB surgery. It is reported that GLP-1 and its long-acting analog exendin-4 (Ex-4) enhance the expression of pancreatic PDX-1 and regeneration of islet β-cells, and inhibit the apoptosis of β-cells[27,28]. GLP-1 can stimulate the differentiation and proliferation of β cells in GK rats[29]. GLP-1 increases the number of β cells by inducing the differentiation and neogenesis of ductal progenitor cells into islet endocrine cells. Koizumi *et al*[27], have found that Ex-4 can significantly stimulate β-cell proliferation in PDX-$1^{+/+}$ mice, but not obviously influence the apoptosis of β-cells. However, in PDX-$1^{-/-}$ mice, the proliferation of β-cells showed no significant change, whereas the apoptosis of β-cells increased significantly in number. Therefore, functions of GLP-1 may rely on the *PDX-1* gene activation and expression[24,30]. Ex-4 has been approved by the Food and Drug Administration for the clinical treatment of diabetes[31]. GLP-1 is decomposed *in vivo* by enzyme dipeptidyl peptidase-Ⅳ (DPP-Ⅳ)[31], whose inhibitor, vildagliptin, has been applied in the treatment of diabetes and is at the stage of advanced clinical trials[30]. This compound may increase the mass of β-cells, and slow or even reverse the progressive islet-cell deterioration in diabetes. It is possible that the effect of GLP-1 on increasing β-cells is partly mediated by an increase in the circulating insulin, which may act as a

growth factor. But, long-term over-stimulation of GLP-1 signaling may cause pathologic hypertrophy and hyperactivity of β-cells, resulting in hypoglycemia.

New pancreatic β-cells are formed in two ways: proliferation of preexisting β-cells, which is called regeneration, and transdifferentiation of non-β-cells from either duct or acinar cells to new β-cells, which is called neogenesis. Dor *et al*[32], have found that the removal of most of the pancreas in mice led to hyperplasia of the pancreas, and suggested that the new β-cells came from the proliferation of pre-existing β cells in the adult pancreas. Injury in the mouse model caused by pancreatic duct ligation has shown that the progenitors of β-cells exist in adult mouse pancreas, which could be activated and then produced new functional β-cells by differentiation and proliferation[33]. It has been reported that new β-cells in adult pancreas were transdifferentiated from epithelial cells in pancreatic ducts whose characteristics as duct cells were lost either in normal or injured pancreas[27,33-37]. Stem cells also existed in adult human pancreatic ducts, which not only expressed nestin and PDX-1, but also exhibited the markers of mesenchymal stem cells[38]. These data suggest that the new β-cells in pancreas may be formed through a variety of channels and derived from different cells.

Similarly, *in vitro* cultured stem cells from adult bone marrows[35] and adipose-derived mesenchymal stem cells[36] can be induced to differentiate into insulin-like cells which produce insulin. This indicates that the stem cells from the mesoderm may cross over the embryonic layer and differentiate into insulin-like cells in the endoderm. In other words, the mesenchymal stem or progenitor cells have a strong reserving function in the body. Yuan *et al*[39] and Kodama *et al*[40] have also proposed similar hypothesis, in which progenitor cells exist in the adult pancreas and may transform to endocrine cells under pathological conditions. In Streptozotocin-treated mice, regeneration of β-cells seemed to occur mainly from intra-islet stem/progenitor cells[40].

In this study, the regeneration of β-cells was mainly located within the islets, which is consistent with the experimental results of Kodama *et al*[40]. In addition, in this experiment, Brdu and insulin double positive cells were mainly found in the pancreatic duct and single insulin positive cells were mainly in the acini. These results suggest that the duct epithelial cells and acinar cells can be transdifferentiated into insulin-like cells, which are consistent with the experimental results of De Haro-Hernández *et al*[41]. Single Brdu positive cells were found within the pancreatic acini in RYGB rats, which suggests that RYGB promotes not only regeneration/neogenesis of pancreatic β-cells, but also regeneration/neogenesis of other cells. However, the emergence of these new cells depends on the PDX-1 activation and expression.

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COMMENTS

Background

Roux-en-Y gastric bypass (RYGB) is a commonly used surgical treatment for patients with morbid obesity. It significantly and persistently decreases the levels of blood glucose and glycosylated hemoglobin in 80%-100% of type 2 diabetes mellitus (T2DM) patients. Efforts have been made to understand the mechanism of RYGB treatment in T2DM patients, however, the exact mechanism is still elusive.

Research frontiers

Some patients who underwent RYGB surgery developed nesidioblastosis, a serious life-threatening postprandial hyperinsulinism with hypoglycemia, which is rarely found in the normal population. The article suggests that RYGB may be associated with the regeneration and neogenesis of the pancreatic islets. However, this hypothesis has not been supported by any animal experiment.

Innovations and breakthroughs

This study showed that RYGB could increase the expression of pancreatic PDX-1, which is the first and most important transcription factor found during the embryonic development of the pancreas and pancreas regeneration, and induce the regeneration of β-cells in GK rats used in a model of genetic non-overweight type 2 diabetes. The regeneration of islet cells is a possible mechanism how RYGB can treat T2DM.

Applications

T2DM is one of the most common chronic metabolic diseases that is characterized by insulin resistance and progressive deterioration of islet cell functions. Findings of this study explain the possible mechanism of treating T2DM with RYGB surgery. This study has provided a new basis for surgery to treat T2DM and explained why the post-surgical nesidioblastosis occurs in RYGB patients.

Peer review

This is a very important paper since it is dealing with an issue of high relevance, nevertheless not properly addressed in most of the publications at present. Moreover, an extensive literature review is reported and examined.

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ORIGINAL ARTICLE

Expression of interleukin 6 in brain and colon of rats with TNBS-induced colitis

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Abstract

AIM: To characterise expression of interleukin 6 (IL-6), a potent proinflammatory cytokine, in the occurrence and development of inflammatory bowel disease (IBD) and investigate its effect on neuroimmunomodulation and immune homeostasis regulation.

METHODS: In this study, rats with colitis induced by trinitrobenzene sulfonic acid (TNBS) were sacrificed on days 3, 7, 14, 21 and 28 after induction. In the controls, the TNBS was just replaced by equivalent amount of phosphate buffered solution (PBS, 0.01 mol/L). IL-6 mRNA expression in brain and colon tissues in each phase was evaluated by real-time reverse transcription-polymerase chain reaction, and cellular localisation and protein level of IL-6 was determined by immunohistochemistry.

RESULTS: At day 7, mRNA expression of IL-6 was significantly higher in the colon and brain of IBD rats than that of the controls. The protein level was also significantly higher in colon, hypothalamus and cerebral cortex of IBD rats compared with the controls. So there are similar temporal trends in IL-6 mRNA expression and protein levels in all positions with a persistent increase to a peak at day 7, followed by a decline and gradual return to normal levels.

CONCLUSION: These results revealed that changes in IL-6 expression in brain and colon tissues occur in different phases of IBD. Therefore, we propose that the nerve centre regulates and controls the occurrence and development of IBD *via* IL-6.

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Key words: Inflammatory bowel disease; Interleukin 6; Neuroimmunomodulation; Real-time transcription-polymerase chain reaction; Immunohistochemistry

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INTRODUCTION

Inflammatory bowel disease (IBD) includes two chronic pathologies characterised by alternating phases of active inflammation and quiescence: ulcerative colitis (UC) and Crohn's disease (CD)[1]. Although the pathogenesis of IBD has not been well established, it has been suggested that some frequently observed clinical manifestations such as abdominal pain, nausea, vomiting, ileus, or diarrhoea, can be attributed to deranged gastrointestinal motility associated with inflammation[2]. The hypothesis that immune abnormalities induced by Th1/Th2 imbalances play a core role in the pathogenesis of IBD has been widely accepted[3,4]. Th1 cells predominantly secrete interferon-γ (IFN-γ), interleukin 2 (IL-2) and tumor necrosis factor α (TNF-α), while Th2 cells secrete interleukins 4, 5 and 10 (IL-4, IL-5, IL-10). Cytokines can be divided into pro-inflammatory and anti-inflammatory cytokines and growth factors. Pro-inflammatory cytokines such as IL-1, IL-2, IL-8, IL-12, TNF-α, TNF-β and IFN-γ are produced by monocytes and macrophages and are involved in cell-mediated immune responses. Anti-inflammatory cytokines such as IL-4, IL-5, IL-10 and IL-13 are produced mainly by T cells and are involved in the humoral immune response.

Communication between the periphery and the brain takes place *via* neural and humoral pathways. Cytokines released by activated immune cells are mediators of inter- and intra-cellular communication[5-11] and are part of a chemical signalling system that regulates development, tissue repair, haemopoiesis, inflammation and specific and nonspecific immune responses. Potent cytokine polypeptides (such as interleukins 1, 6, 8 and TNF-α) have pleiotropic activities and functional redundancy and exist in a complex, intermingled network in which one cytokine can influence the production of and response to many other cytokines[12]. Of note, modulation of cytokines and their antagonists also exhibits therapeutic potential in several chronic and acute diseases[10,11]. The immune system is regulated in part by the central nervous system (CNS), acting principally *via* the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system (SNS)[13-15]. IL-6 is a pleiotropic cytokine whose expression in physiological conditions is important for host responses to a number of infections; in addition, it exerts antigen-specific immune responses and has both pro- and anti-inflammatory effects[16,17]. In CD, IL-6 is present at high levels in both serum and intestinal tissues[18]. Increased levels of IL-6 receptor (IL-6R) and gp130 expression have been also demonstrated in peripheral lymphocytes of Crohn's patients, along with increased serum soluble IL-6R[19].

To explore the role of IL-6 in the initiation and development of IBD with respect to the neuroimmunomodulation and regulation of immunological homeostasis at the organismic level, we used real-time reverse transcription polymerase chain reaction (RT-PCR) and immunohistochemistry (IHC) to evaluate the mRNA expression and cellular localisation of IL-6 in brain and colon tissues in the inflammatory and quiescent phases of IBD in an animal model with trinitrobenzene sulfonic acid (TNBS)-induced IBD.

MATERIALS AND METHODS

Reagents

All chemicals were purchased from Sigma Chemical, unless otherwise stated.

Animals

Eighty female Wistar rats (Laboratory Animal Service of Jilin University, Changchun, China), 8-10 wk old and weighing 180-200 g, were maintained under specific pathogen-free conditions according to our institutional guidelines for animal care. This study was carried out in accordance with guidelines established by a consensus statement entitled the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes.

Experimental model

After a seven-day acclimation period, 80 rats were divided randomly into 8 cages (one cage for 10) and fed in the same condition. Among them, 4 cages were experimental group and the others were controls. Rats were fasted overnight, and colitis was induced in the TNBS group by the method originally described by Morris *et al*[20], with some modifications made by our group. Briefly, rats were anaesthetised with ethylether and given a 5% TNBS (Sigma, R&D, USA) and ethanol mixture (2:1) at a dose of 0.3 mL/100 g inserted through the anus to a distance of 8 cm. Rats from the non-colitis group were given an intracolonic dose of 0.25 mL phosphate buffered saline (PBS) instead of TNBS. Animals were maintained in a vertical position for 30 s and returned to their cages.

Assessment of experimental model

Macroscopic scoring: At 3, 7, 14, 21, and 28 d after induction of colitis, rats were exsanguinated under ether anaesthesia, and the small and large intestines and the brain were excised. Samples of the descending colon were obtained for paraffin section and measurement of myeloperoxidase activity (see below). The remaining descending colon, sigmoid colon, and rectum were opened longitudinally and fixed onto a board with pins, and the macroscopic appearance was scored by two blinded investigators on a 0-4 scale similar to the original scoring system of Morris and colleagues[20]; briefly, 0 = no evidence of inflammation, 1 = erythema only, 2 = erythema with slight oedema and small erosions, 3 = two or more bleeding ulcers and/or inflammation and/or moderate adhesions, and 4 = severe ulceration and/or stenosis with prestenotic dilations with or without severe adhesions.

Determination of myeloperoxidase activity: Colonic myeloperoxidase activity was measured as previously described by Grisham *et al*[21] and Suzuki *et al*[22]. Briefly, tissue was homogenised in approximately 10 mL 50 mmol/L

KPO_4 at pH 7.4 and centrifuged for 20 min at 20 000 *g*. The pellet was homogenised in 10 mL 50 mmol/L KPO_4, and 10 mmol/L EDTA at pH 6.0 with 0.5% hexadecyltrimethylammonium bromide (wt/vol). After one freeze-thaw cycle, the homogenate was sonicated, and myeloperoxidase activity was determined by measuring H_2O_2-dependent oxidation of 3,3',5,5'-tetramethylbenzidine and expressed as units per gram of tissue.

Microscopic scoring: Specimens were fixed in PBS containing 4% formaldehyde and embedded in paraffin. Multiple sections (5 μm thick) were stained with haematoxylin and eosin using standard techniques. The degree of inflammation was assessed by two blinded investigators using the 0-4 scoring system described by Neurath *et al*[23] (0 = no signs of inflammation, 1 = low levels of leucocyte infiltration, 2 = moderate levels of leucocyte infiltration, 3 = high levels of leucocyte infiltration, high vascular density, thickening of bowel wall, and 4 = transmural infiltrations, loss of goblet cells, high vascular density, significant bowel wall thickening).

Isolation of mRNA and analysis of mRNA expression by real-time RT-PCR

A total of 80 rats (normal and IBD model) were used for real-time RT-PCR analysis. The proximal part of the colon (the area with macroscopically visible mucosal folds) and the brain were stored in RNAlater (Ambion, Austin, TX) at -80℃ for use in RNA extraction. RNA was extracted using the Trizol reagent (Invitrogen) according to the manufacturer's protocol. The integrity and quality of the isolated RNA was examined by quantifying the A260/280 ratio (BioPhotometer, Eppendorf, Hamburg, Germany) and resolving the RNA on a 1% agarose gel. The cDNA was prepared from 1.0 μg RNA (OD = 1.9-2.0) in the presence of 2.5 μmol/L oligo (dT) primer and 200 U Moloney murine leukemia virus reverse transcriptase (M-MLV; Promega, Madison, WI) in a total volume of 20 μL. The reaction mixture was incubated for 1 h at 42℃ and stopped by heating at 90℃ for 5 min. The primers and FAM-labelled probes were designed with Primer Express v1.5 software (Applied Biosystems). For IL-6, the forward primer was 5'-GCCCTTCAGGAACAGCTATGA-3', the reverse was 5'-TGTCAACAACATCAGTCCCAAGA-3', and the probe was 5'-CTCTCCGCAAGAGACTTCCAGCCAGTT-3'. For the housekeeping gene beta-actin, the forward primer was 5'-GTCAGGTCATCACTATCGGCAAT-3', the reverse was 5'- AGAGGTCTTTACGGATGTCAACGT-3', and the probe was 5'-CCTTCCTTCCTGGGTATGGAATCCTGTG-3'. All the primers and probes were obtained from Applied Biosystems. PCR was performed in the ABI PRISM 7000 Sequence Detection operated by Sequence Detector v1.3 software (Applied Biosystems), using TaqMan Real-time PCR Master Mix (Toyobo, Japan). A threshold was set at the linear part of the amplification curve, and the number of cycles needed to reach it were calculated. Relative mRNA levels were determined by using a standard curve and by further normalisation to the housekeeping gene beta-actin. The annealing temperatures were both set to 60℃.

Immunohistochemistry

IHC for detection of IL-6 was performed on paraffin sections of rat brain and colon samples; female rats were perfused under chloral hydrate anaesthesia *via* the abdominal aorta. After a 30 s rinse with physiological saline, a fixative containing 4% freshly prepared paraformaldehyde in 0.01 mol/L PBS buffer (pH 6.0) was perfused for 5 min. The brains and colons were cut into 1-2 mm frontal or sagittal slices and were embedded in paraffin using an automated vacuum tissue processor (Leica ASP300, Germany). For experiments, 4 μm sections were cut and mounted on specific slides. For antigen retrieval, the protocol recently described by Pizarro *et al*[24] and Strober *et al*[25] was used. Briefly, the deparaffinised and rehydrated sections were subjected to a 5 min digestion with 0.01% trypsin (Sigma) in PBS and an additional microwave treatment in 10 mmol/L citrate buffer (pH 6.0) for 15 min at 720 W, followed by a 20 min cooling period in the same buffer. After pretreatment, sections were treated for 15 min with 3% hydrogen peroxidase to block endogenous peroxidase activity and were blocked with 0.5% blocking reagent for 30 min to reduce non-specific reactions. The sections were incubated for 36 h at 4℃ with rabbit anti-rat-IL-6 IgG (Biozol, Eching, Germany; diluted 1:400) in blocking buffer. Sections were washed in PBS, and biotinylated linking antibody solution (Biozol, Eching, Germany) was applied (100 μL/section) for 20 min at 37℃. Sections were then washed in PBS before application of HRP conjugated streptavidin (100 μL/section, 15 min) at 37℃. Slides were stained with DAB (IBL, Germany) after being washed in PBS for 5 min, washed in PBS and DAB (BIOS) for 5 min and counterstained with hematoxylin. For negative controls, the primary antibody was replaced with the corresponding affinity-purified preimmune IgG.

Statistical analysis

Data are expressed as mean ± SE. Statistical analysis was performed using SPSS 17.0. Differences were considered to be statistically significant when $P < 0.05$ by analysis of variance (ANOVA).

RESULTS

Effects of TNBS-induced colitis on clinical and macroscopic appearance

After administration of TNBS, we found that Wistar rats regularly developed colitis with severe diarrhoea, losing 15% of their body weight and suffering rectal prolapse and extensive wasting. Gross blood adhesion to the anus was noted in some rats. Macroscopically, colitis was characterised by bowel wall thickening, adhesions and necrotic foci (Figure 1). As these severe inflammatory changes covered 2-4 cm of the distal colonic mucosa, this resulted in a higher median macroscopic damage score in TNBS-treated rats compared to controls (Figure 2A, $P < 0.01$), especially by day 7.

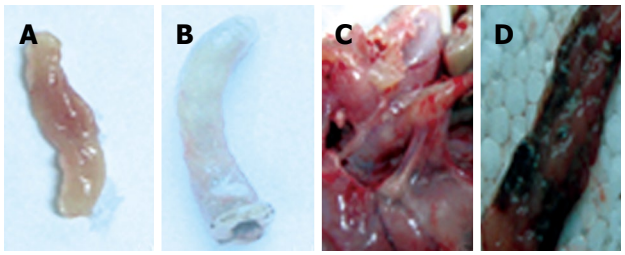


Figure 1 The macroscopic appearance of colon tissue in trinitrobenzene sulfonic acid (TNBS)-treated *vs* control rats. A: The colon of control rats, with macroscopic damage score = 0; B-D depict colonic tissue of TNBS-treated rats: B: Significant swelling and distension, with macroscopic damage score = 5; C: Extensive adhesions around the colon, with macroscopic damage score = 7; D: Necrotic foci in colon, with macroscopic damage score = 8.5.

Effects of TNBS-induced colitis on colonic myeloperoxidase activity

Myeloperoxidase (MPO) activity was determined by using accumulation of polymorphonuclear leucocytes as an indicator. Compared with the control rats, MPO activity increased gradually, peaked by day 7, and decreased to nearly the baseline value (Figure 2B).

Effects of TNBS-induced colitis on colonic histology

The degree of inflammation was assessed using the scoring system at 3, 7, 14, 21, and 28 d after TNBS induction. The score reached a peak at day 7, and then decreased gradually to nearly the normal value (Figure 2C). Microscopic evaluation of colons from control rats showed normal colonic histological features (Figure 3A). Histological evaluation of the colon at each period after TNBS induction is shown in Figure 3B-F. TNBS caused the inflammatory features typical of colitis in the rats' colonic architecture: severe oedema and haemorrhage at the muscular layer of the mucosa, ulceration, goblet cell depletion, and a mixed cell infiltration that was mainly composed of mononuclear cells such as macrophages, lymphocytes and plasmocytes.

Analysis of IL-6 mRNA expression

We used real-time RT-PCR to measure expression of IL-6 mRNA in the brain and colon at various time points after induction of colitis. Our results demonstrated a remarkable difference ($P < 0.01$) in colon and brain tissues in the experimental *vs* the control group. IL-6 mRNA expression levels increased by day 3 and returned to near baseline levels by day 28, reaching their highest point at day 7. Thus, there is similarity on the increasing trend (Figure 4A).

IHC

IHC was performed to determine IL-6 localisation in the colon, cerebral cortex and hypothalamus. The distribution, cellular localisation, and intensity of staining were assessed using light microscopy. The distribution and relative abundance of IL-6 in the parts of the colon, cerebral cortex and hypothalamus examined in this study are presented in Figure 4B; representative sections are shown in Figure 5. Control sections immunolabeled with the preimmune serum were devoid of immunolabeling for IL-6. We found that

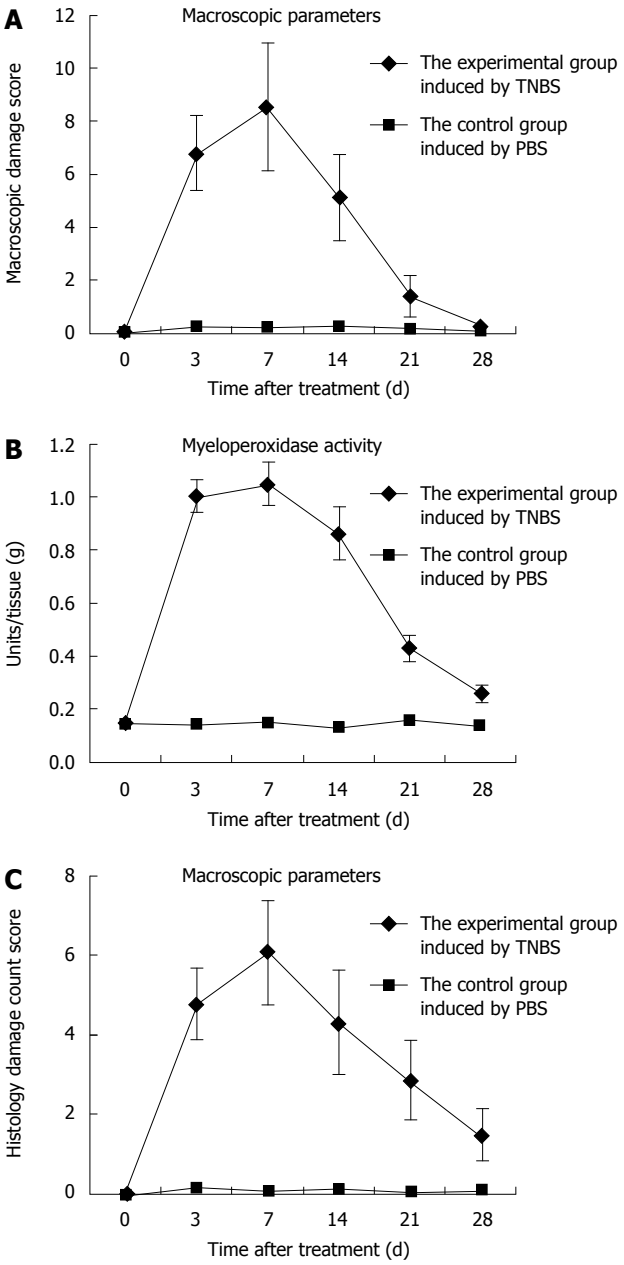


Figure 2 Assessment of TNBS-induced colitis rats by time points. As these severe inflammatory changes covered 2-4 cm of the distal colonic mucosa, this resulted in a higher median macroscopic damage score in TNBS-treated rats compared to controls (A, $P < 0.01$), especially by day 7; compared with the control rats, MPO activity increased gradually, peaked by day 7, and decreased to nearly the baseline value (B); The score reached a peak at day 7, and decreased gradually to nearly the normal value (C).

immunostaining was clearly increased in the colon, cerebral cortex and hypothalamus after induction of colitis, reaching a peak at day 7 and decreasing thereafter. Morphologically, analyses showed that the number of IL-6 immunopositive cells was significantly increased ($P < 0.01$) in the colon, cerebral cortex and hypothalamus after induction compared to control animals.

DISCUSSION

Over the past several years, a variety of IBD animal mod-

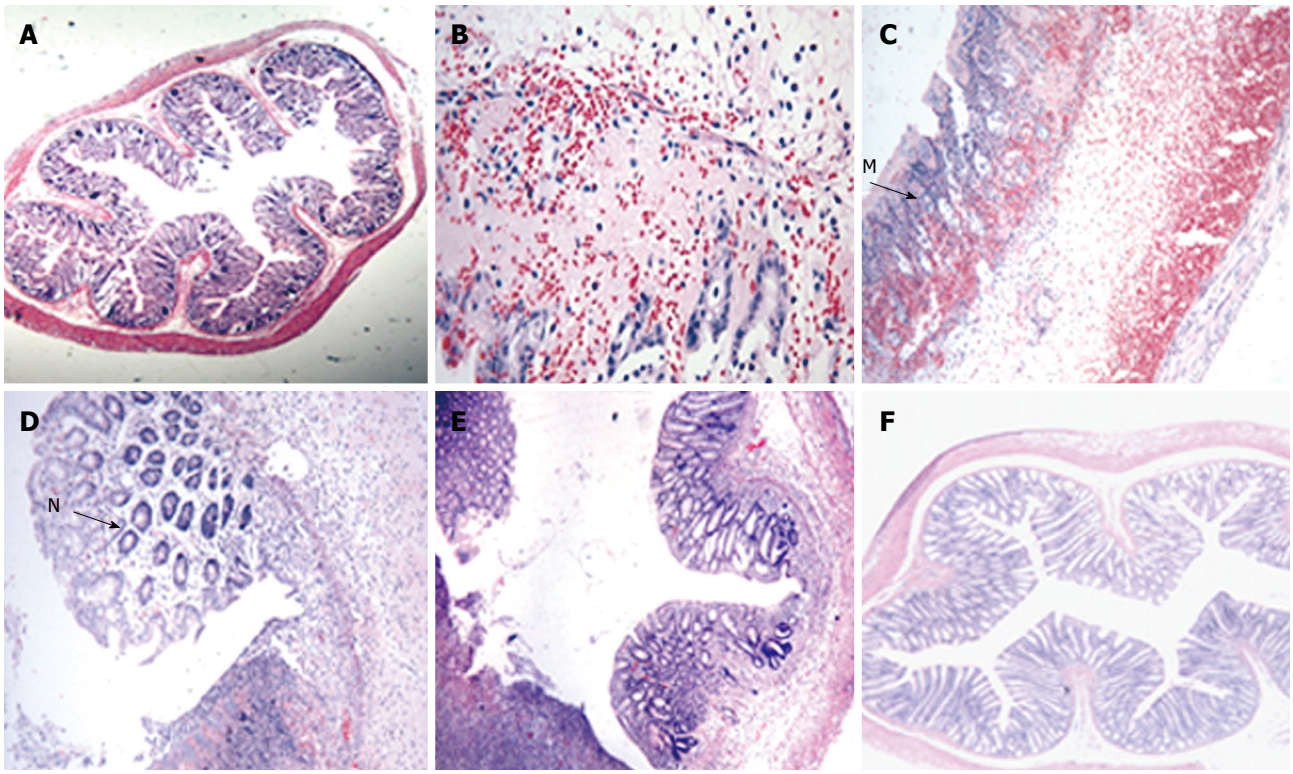


Figure 3 Light photomicrograph of colon tissues from TNBS-induced colitis rats. In control rats, normal histological colon morphology is evident (A, × 4). At day 3, oedema and haemorrhage at the mucosa with goblet cell depletion were seen (B, × 20). At day 7, severe transmural infiltration of the colon wall developed, with submucosal oedema and haemorrhage, lymphocytic and neutrophilic infiltration (M) and coagulative necrosis (C, × 10). At day 14, goblet cell regeneration (N), oedema and haemorrhage regressed by degrees (D, × 20). At day 21, abundant goblet cells and crypts developed (E, × 10). At day 28, oedema and haemorrhage had almost entirely disappeared, the structure of the muscular layer became compact and the intestinal glands developed highly (F, × 4).

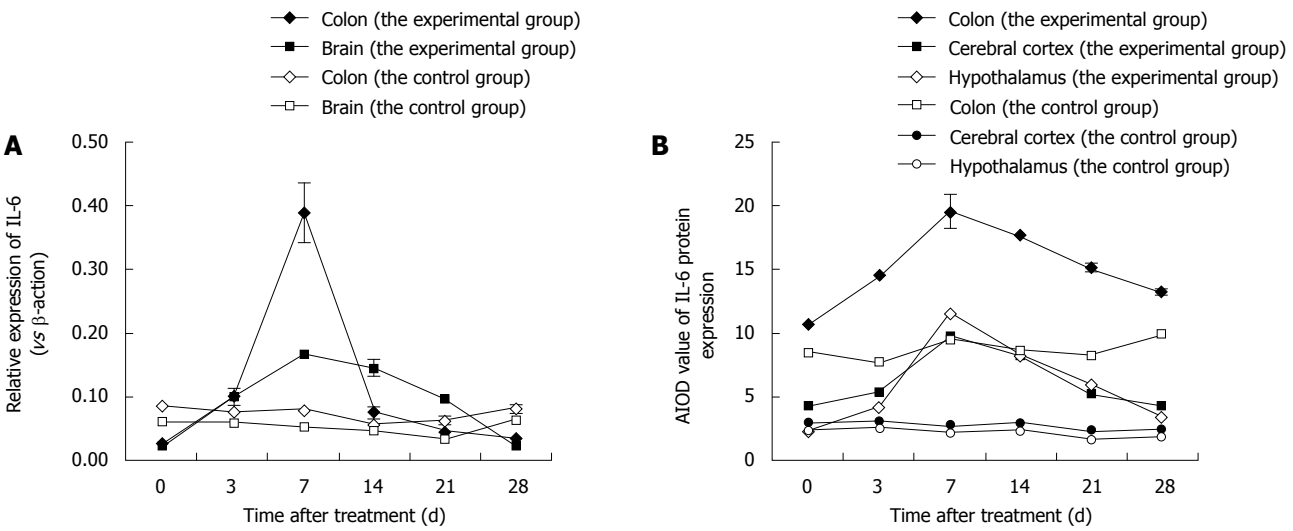


Figure 4 Variation in expression of interleukin-6 (IL-6) in the colon and brain of TNBS-induced colitis rats by time points (at days 3, 7, 14, 21 and 28) *vs* control rats. A: The expression of IL-6 mRNA after real-time RT-PCR; Data shown as mean ± SD, n = 4-8; B: The average integrated optical density (AIOD) values for IL-6 protein expression after immunohistochemical staining. The results were determined by ANOVA.

els have been developed and investigated, with the ultimate goal of understanding the causes of CD and UC[24,25]. To study the inflammatory pathways and pathogenesis of IBD, we have established models of colitis after chemical induction (TNBS) of hapten-induced gut inflammation; with this model, we have detected mRNA expression and cellular localisation of IL-6 using RT-PCR and IHC in brain and colonic tissues.

Our IHC and RT-PCR analysis of the colon demonstrate an important role for IL-6 in the development of IBD. The induction-related changes in IL-6 described here are not limited to our animal models and are seen in human IBD as well[26,27]. Although the pathogenesis of IBD is unclear, many earlier experiments have proved that IL-6

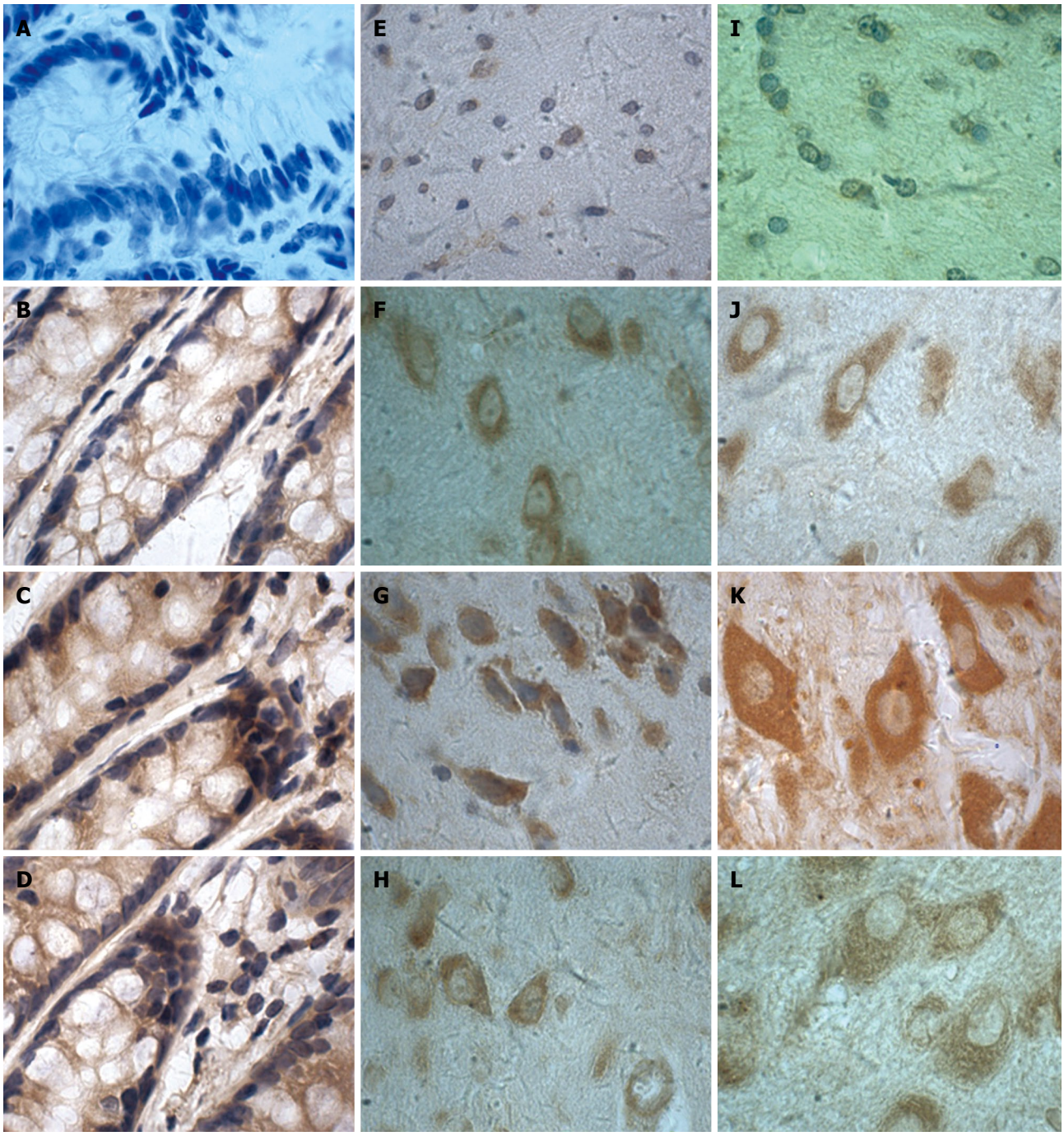


Figure 5 Immunohistochemical localisation of IL-6 proteins in colon (A-D), cerebral cortex (E-H) and hypothalamus (I-L) from TNBS-induced colitis (× 100). C, G, K: Day 7; D, H, L: Day 28; B, F, J: Day 0; A, E, I: Negative control (the primary antibody was replaced with the corresponding affinity-purified preimmune IgG).

plays an important role in the course of colitis[28,29]. IL-6 is one of several inflammatory mediators that induce lesions in the intestinal mucosa; however, if a small amount is generated, the organism's immune function may be enhanced (particularly anti-viral, anti-infectious and anti-tumour activities), while excessive production of IL-6 may induce septic shock and pyosepticaemia. IL-6 expression in CD and UC has been found to be increased[30]. Using RT-PCR, Ishiguro *et al*[31] found enhanced mRNA expression of IL-1β, IL-6, IL-8 and TNF-α in CD and UC, as well as a positive correlation of IL-6 expression levels with severity of inflammation. These results showed that when pathological changes in intestinal canal were increased, IL-6 expression in colonic tissues was gradually enhanced; with the alleviation of pathological changes in the intestinal canal, colonic IL-6 expression gradually declined and tended towards normal. It should be noted that inflammatory cells secreting IL-6 were present in intestinal inflammation sites, and their number was closely related to the severity of inflammation.

There is increased evidence to support the hypothesis of bidirectional circuits between the immune system, CNS and endocrine system[12]. Neuroimmunomodulation is one of the fastest-growing fields of study in current biomedi-

cal science. There is also abundant evidence suggesting that the CNS is not an immune-privileged area and that T lymphocytes are present in the CNS. Microglial cells and astrocytes in the CNS that are equivalent to macrophages in other tissues are the primary cells responsible for an inflammatory reaction. In normal cerebral tissues, these glial cells are quiescent, but they are highly reactive and can secrete cytokines rapidly; this is followed by changes in internal cellular environments and in cell-surface antigens. Cytokines produced by the CNS, including interleukins 1-24, TNF and TGF, can reduce the production of proinflammatory cytokines, lowering the occurrence of some pathological behaviours regulated by the CNS[32,33]. Thus, cytokines may not only play an important role in the immune system, but may also show wide-ranging central regulatory effects. Together with neuromediators and endocrine hormones, cytokines act as the intercellular signalling molecules in organisms and are involved in immune activation and information transmission using neurotransmitters and hormones; thus they are closely related to psychological reactions and mental disorders. In-depth studies on cytokines will contribute to our understanding of IBD pathogenesis and potential therapeutic options. Because neural cells produce IL-6 and have IL-6 receptors, several studies have suggested that IL-6 plays a role in regulating their response to stress. Moreover, IL-6 may be an essential neurotrophic factor in the midbrain after long-term stress[34]. Loss of IL-6 in the CNS does not lead to an improvement in memory function, but instead to memory impairment[35]. Moreover, IL-6 trans-signalling may be of paramount importance in the pathogenesis of several immune-mediated disorders, as well as diseases of the CNS[36,37]. IL-6 is an important CNS-related cytokine and is involved in disease occurrence and course, by direct or indirect regulation of neuronal excitability and neurotransmitter release after receptor binding. IL-6 also plays a significant role in glial cell differentiation and proliferation and reduces antigen presentation by glial cells in the CNS[38]. It is currently recognised that neurons and glial cells can also secrete and express cytokines, and cytokines are active during nerve growth and in the adult nervous system under normal and pathological conditions. This evidence speaks for a potential and crucial role for IL-6 in regulating the interaction of the immune system, CNS and endocrine system. We have confirmed this conclusion through the detection of IL-6 expression in the brains of IBD rats, using IHC and RT-PCR.

Over the course of IBD, we found that expression of IL-6 in cerebral cortex and hypothalamus increased when IBD was exacerbated and gradually declined with disease alleviation. Neither cytokines in cerebral tissues nor those in peripheral blood can cross the blood-brain barrier[39], and there are no reports of an association between cerebral IL-6 expression and colonic cytokines or of what their relationship might be in IBD. The CNS is a central link in the nerve-endocrine-immune regulation network and plays a leading role in the maintenance of the nerve-endocrine-immune network homeostasis. Therefore, by exploring the role of IL-6 in IBD rats from the perspective of the nerve-endocrine-immune network and homeostatic regulation, we have explored the general role of the nerve-endocrine-immune network over the course of IBD. Our results show that pathological changes were most obvious and severe at day 7 after induction of colitis, with necrosis and exfoliation of the intestinal epithelial cells and severe oedema and haemorrhage in the mucosa and muscularis layers, as well as increased expression of IL-6 in the cerebral tissues. Conversely, with the resolution of enteropathy and alleviation of symptoms, IL-6 expression in cerebral tissues began to decline and gradually trended towards levels in normal rats. Therefore, there may be a correlation between IL-6 expression in cerebral and colonic tissues. We propose that cerebral tissues may regulate and control the occurrence and development of IBD *via* IL-6.

This experiment provides new evidence concerning IBD pathogenesis, as well as an experimental model for further enriching and improving the nerve-endocrine-immune network theory. Future studies that explore signal transduction of IL-6, e.g. in IBD rats, may help to further elucidate its role in neuroimmunomodulation.

COMMENTS

Background

Since inflammatory bowel disease (IBD) appeared in Northern Europe and North America, the incidence has been rising in these areas. Currently the incidence of IBD is increasing rapidly in Asian countries especially in the last two decades. Although considerable progress has been made, a major gap in knowledge of the pathogenesis of IBD remains and has precluded the discovery of lasting, effective forms of therapy.

Research frontiers

It has become increasingly evident that interactions between the nervous system and the immune system play an important role in the pathophysiology of IBD. However, how neuroimmunomodulation plays its role in the occurrence and development of IBD is unknown. In this study, the authors demonstrate that interleukin-6 (IL-6) could play a potential role in the course of colitis.

Innovations and breakthroughs

Recent reports have highlighted the importance of cytokines, including pro- and anti-inflammatory cytokines, in the development of IBD. In particular, the imbalance of the cytokines can lead to improper immune responses and initiation of the inflammatory process. So the authors demonstrated similar trends of IL-6 in the brain and in the colon of the experimental IBD model.

Applications

By understanding the role of IL-6 in the development of IBD, this study may open a new strategy for research into the pathogenesis, prevention and treatment of the autoimmune colitis.

Peer review

This is simple and solid work with some new and interesting results.

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BRIEF ARTICLE

Covered nitinol stents for the treatment of esophageal strictures and leaks

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Abstract

AIM: To compare 2 different types of covered esophageal nitinol stents (Ultraflex and Choostent) in terms of efficacy, complications, and long-term outcome.

METHODS: A retrospective review of a consecutive series of 65 patients who underwent endoscopic placement of an Ultraflex stent (n = 33) or a Choostent (n = 32) from June 2001 to October 2009 was conducted.

RESULTS: Stent placement was successful in all patients without hospital mortality. No significant differences in patient discomfort and complications were observed between the Ultraflex stent and Choostent groups. The median follow-up time was 6 mo (interquartile range 3-16 mo). Endoscopic reintervention was required in 9 patients (14%) because of stent migration or food obstruction. No significant difference in the rate of reintervention between the 2 groups was observed (P = 0.8). The mean dysphagia score 1 mo after stent placement was 1.9 ± 0.3 for the Ultraflex stent and 2.1 ± 0.4 for the Choostent (P = 0.6). At 1-mo follow-up endoscopy, the cover membrane of the stent appeared to be damaged more frequently in the Choostent group (P = 0.34). Removal of the Choostent was possible up to 8 wk without difficulty.

CONCLUSION: Ultraflex and Choostent proved to be equally reliable for palliation of dysphagia and leaks. Removal of the Choostent was easy and safe under mild sedation.

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Key words: Dysphagia; Esophageal neoplasms; Endoscopy; Palliative care; Surgical anastomosis; Stricture; Neoadjuvant therapy; Self-expanding metal stents

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INTRODUCTION

Less than 50% of patients with esophageal carcinoma are suitable for surgery at the time of diagnosis. Most of these patients present with locally advanced or metastatic disease and/or significant comorbidities. In such circumstances, the only therapeutic option is palliative care to treat dysphagia and prevent respiratory complications secondary to aspiration.

Over the past 20 years, the use of covered self-expanding metal stents (SEMS) has proven effective in

patients with unresectable esophageal carcinoma and in those with a tracheo-esophageal fistula[1-3]. In addition, covered SEMS have been successfully used in the management of patients with anastomotic leaks or fistulas[4,5]. Despite the large number of different covered metal stents available on the market, the superiority of one type over another has not yet been proven[6,7]. The aim of this study was to review our experience with 2 types of covered nitinol stents in the management of patients with esophageal strictures and anastomotic complications.

MATERIALS AND METHODS

From July 2001 to October 2009, 422 patients with esophageal stricture due to cancer were seen at our institution. A total of 263 (62.3%) patients had surgical resection with or without neoadjuvant chemotherapy or chemoradiotherapy. We retrospectively reviewed the charts of 65 consecutive patients who underwent endoscopic placement of a covered nitinol stent. Two types of stents were used: Ultraflex (Boston Scientific, MA, USA), and, more recently, Choostent (M.I. Tech, Seoul, Korea). Ultraflex stents had a diameter of 18 or 23 mm and a length of 10 or 12 cm; Choostent stents had a diameter of 18 mm and lengths of 8, 11, 12 or 14 cm.

The indications for placement of the stent are shown in Table 1. The grade of dysphagia at presentation was defined using a 0 to 4 grading system as shown in Table 2. Demographic and clinical data are presented in Table 3.

Assessment of the extent of disease included a videoesophagram, upper endoscopy with biopsy, chest and abdominal computed tomography scan. In a subset of patients, total body positron emission tomography was performed to rule out the presence of distant metastases. If the tumor was in close proximity with the carina, a fiber-optic bronchoscopy was performed to evaluate the presence of infiltration and/or compression that could affect ventilation after placement of the stent. In some patients, Savary esophageal dilators were used to simulate the presence of the stent during the bronchoscopic examination. Written consent for the treatment was obtained from all patients.

Prior to the procedure, an antibiotic (ceftazidime 1 g iv) was administered to the patient. Endoscopic stent placement was performed with fluoroscopic guidance in the operating room, with the patient in left lateral decubitus. In 95% of the cases, conscious sedation with midazolam was used; in 3 patients propofol was required to achieve an adequate level of sedation. If the diameter of the lumen was too small to allow placement of the stent, dilatation up to 9 mm in diameter was performed with Savary bougies.

In patients with tumors of the thoracic esophagus, the proximal and distal margins of the lesion were marked with water-soluble ink injected into the submucosa. In patients with tumors located at the gastroesophageal junction, only the proximal margin was marked. In a few cases the proximal and distal limits of the tumor were marked using metallic endoclips[8]. Complete opening of the stent was expected within 48-72 h after implantation. In most patients, no attempt was made to pass the stent with the endoscope after its deployment in order to prevent migration.

Table 1 Indication for use of the stent in 65 patients

	n (%)
Esophageal carcinoma	51 (78.5)
Adenocarcinoma	31
Squamous-cell carcinoma	20
Complications of esophagogastric anastomosis	8 (12.3)
Fistula	3
Stricture	3
Tumor recurrence	2
Extrinsic compression	4 (6.1)
Lung cancer	2
Pleural mesothelioma	1
Mediastinal lymphoma	1
Post-radiotherapy stricture	2 (3.1)

Table 2 Classification of dysphagia

Grade	Definition
0	Normal swallowing
1	Able to swallow some solid food
2	Able to swallow semi-liquid food
3	Able to swallow liquids only
4	Absolute dysphagia

After the procedure, a non-steroidal antiinflammatory (ketorolac) and proton pump inhibitors (e.g. pantoprazole) were administered intravenously. Chest X-ray and a gastrografin swallow study were performed the day after stent placement. Patients were then allowed to eat a semi-liquid diet until discharge.

RESULTS

Stent placement was technically successful in all patients. Nine patients (13.8%) presenting with severe stricture required preliminary dilation before deployment of the Ultraflex (n = 5) or the Choostent (n = 4). The procedure took a mean of 16 min (range, 12-35 min) with the Ultraflex, and 17 min (range, 13-27 min) with the Choostent (P = 0.8). There were no deaths related to the procedure. Periprocedural complications occurred in 4 patients (6.1%): 2 had fever probably related to aspiration pneumonia, 1 had an episode of atrial fibrillation managed with amiodarone iv, and 1 had acute urinary retention requiring catheterization. The 2 types of stent showed equal palliative efficacy against dysphagia. Most patients were discharged within 48 h. The results of the treatment are summarized in Table 4.

Early and late post-procedural complications are shown in Table 5. Severe chest pain immediately after stent insertion was present in 3 patients who had an Ultraflex implanted. The pain disappeared within 36 h of iv infusion of morphine. Overall, 9 patients (14%) needed a second endoscopic intervention. In 1 patient of the

Table 3 Demographic and clinical data of the patient population

	Total	Ultraflex	Choostent	*P*-value
Patients	65	33	32	-
Male/female	43/22	23/10	20/12	0.5
Mean age (range)	67.5 (34-86)	66.8 (34-83)	68.3 (42-86)	0.6
Grade of dysphagia (mean ± SD)	3.4 ± 0.4	3.4 ± 0.3	3.5 ± 0.4	0.9
Diagnosis				
Adenocarcinoma	31	16	15	0.9
Squamous cell carcinoma	20	11	9	0.8
Extrinsic compression	4	2	2	0.9
Anastomotic fistula	3	2	1	0.6
Anastomotic stricture	3	1	2	0.5
Anastomotic recurrence	2	1	1	0.9
Post-radiotherapy stricture	2	0	2	0.1
Site of lesion				
Cervical	3	2	1	0.6
Upper thoracic	9	6	3	0.3
Middle thoracic	21	11	10	0.9
Lower thoracic	32	14	18	0.4
Tumor stage (n = 51)				
Ⅲ	19	12	7	0.6
ⅣA	17	8	9	0.7
ⅣB	15	7	8	0.5

Table 4 In-hospital characteristics and long-term outcome after stent placement

	Ultraflex (n = 33)	Choostent (n = 32)	*P*-value
Duration of the procedure (min)	16 (12-35)	17 (13-27)	0.8
Median hospital stay (d)	2 (2-7)	2 (2-4)	0.7
Hospital mortality (%)	0	0	1.0
Hospital morbidity (%)	6.1 (2/33)	6.2 (2/32)	0.8
Pain score (scale 0-10)	6.3	4.8	0.2
Residual dysphagia (grade)	1.9 ± 0.3	2.1 ± 0.4	0.6
Mean survival (mo)	6.5 (1-19)	6 (3-26)	0.7

Table 5 Early and late complications after stent placement

	n (%)	Ultraflex (n = 33)	Choostent (n = 32)	*P*-value
Abrasion of soft palate	1 (1.5)	1	0	0.3
Odynophagia	1 (1.5)	0	1	0.3
Malposition	1 (1.5)	0	1	0.3
Late distal migration	3 (4.6)	2	1	0.6
Persistent chest pain	3 (4.6)	2	1	0.6
Persistent hiccups	3 (4.6)	1	2	0.6
Gastroesophageal reflux	14 (22)	7	7	0.9
Food obstruction	5 (7.6)	2	3	0.6

Choostent group, the radiographic control showed malpositioning of the stent (too distal release) thus requiring the insertion of a second device overlapping the first one. No stent migration was observed within 72 h after starting oral intake. Interestingly, symptomatic gastroesophageal reflux occurred in 14 (43.7%) of the 32 patients with a stent placed in the lower esophagus.

Upper gastrointestinal endoscopy was performed 1 mo after the procedure in 21 patients with the Ultraflex and in 19 patients with the Choostent. None of these individuals were complaining of dysphagia. The cover membrane of the Choostent appeared to be damaged more frequently compared to the Ultraflex (26% *vs* 14%, $P = 0.34$).

Satisfactory palliation of dysphagia was achieved also in patients with stricture of the esophagogastric anastomosis, and post-radiotherapy stricture. In the majority of these individuals the Choostent was easily removed 3 to 4 wk after the insertion under mild intravenous sedation. One of the 2 patients with an Ultraflex stent required general anesthesia for removal because of a marked tissue reaction and embedding of the proximal edge of the stent.

The worst clinical outcome was recorded in patients suffering from extrinsic malignant compression. One of these patients with dysphagia caused by a bronchial carcinoma died because of massive bleeding 21 d after stent placement. The other 3 patients did not achieve complete palliation of dysphagia and died within 2 mo because of progression of the underlying disease.

The stenting procedure was effective in 2 of the 3 patients with fistula of the esophagogastric anastomosis. The stent was successfully removed in all patients after a mean of 4 wk. Radiological evaluation showed persistent leakage in 1 patient who required insertion of another stent.

Twenty-six of the 65 patients (40%) received chemotherapy or chemoradiotherapy after stent implantation. In 7 patients, a Choostent was uneventfully removed under mild sedation within 8 wk from the beginning of chemotherapy and oral intake was well tolerated. Three of these patients showed significant down-staging of the disease that eventually allowed esophagectomy to be performed without complications.

The incidence of mechanical complications requiring further endoscopic intervention after stent implantation was similar in patients treated or not with chemotherapy or chemoradiotherapy. In treated patients (n = 26) there was an 11.5% incidence of stent dislocation, whereas in

patients who did not receive additive treatment (n = 31) there was a 12.9% incidence of food obstruction.

DISCUSSION

Self-expanding metal stents came onto the market at the beginning of the 1990s and gradually replaced the old Celestin tube. The endoscopic placement of SEMS has proven to be technically easier, requiring minimal dilatation, and resulting in less morbidity and better palliation of dysphagia. In addition, SEMS provide a better quality of life[9], particularly for patients with a Karnofsky index greater then 50[10]. The efficacy of these stents, the ease of insertion, and the large spectrum of diameters and lengths available has resulted in their widespread use also in patients with anastomotic leaks[11].

The standardization of the endoscopic technique and the precise placement mechanism have reduced, but not eliminated, the rate of intraoperative complications. Late complications range from 26% to 52%, especially in patients with adenocarcinoma, and complications requiring additional intervention are frequent[12]. The choice of the correct stent diameter in each patient may represent an important factor for the success of the procedure. The use of an Ultraflex stent with a large diameter significantly reduced the chances of recurrent dysphagia, formation of granulation tissue, and the risk of food obstruction compared to other SEMS[13]. Despite the reported high incidence of covered stent migration at the gastroesophageal junction[14], we believe this is still the best available palliative option in these patients. However, the overall incidence of symptomatic gastroesophageal reflux was 22% in our series, and was almost double (43.7%) among the 32 patients with a stent placed in the lower esophagus. Theoretically, the use of stents provided with an antireflux valve can prevent gastroesophageal reflux, thereby avoiding the risk of aspiration pneumonia[15].

To date, no significant differences in outcomes or complication rates have been reported with the available covered SEMS. The present study shows that there are no statistically significant differences between the Ultraflex and the Choostent, although insertion of the Choostent in the oro-pharynx was less traumatic and post-procedural pain was reduced in the Choostent group. In general, we found it more convenient to use the Ultraflex in patients with strictures of the proximal esophagus because of the ease of stent application under visual control (proximal release). In contrast, the distal release mechanism of the Choostent still allows the operator to modify the site of delivery before the stent has reached 50% of the maximum diameter. Interestingly, a greater frequency of degeneration of the covering film was observed at the 1-mo follow-up endoscopy in the Choostent group compared to the Ultraflex group.

Another finding of this study is that temporary stent placement has indeed a role in patients undergoing neo-adjuvant therapy and in those with anastomotic complications or post-radiotherapy strictures. In such circumstances, the complete cover membrane of the Choostent may cause less granulation tissue and allows easier removability a few weeks later. The benefit of temporary stent insertion has been suggested as a "bridge" to surgery in patients undergoing neo-adjuvant therapy[16]. This minimally invasive and reversible treatment can represent an alternative to trans-nasal feeding tube placement, endoscopic percutaneous gastrostomy, or jejunostomy. Stent placement is usually better accepted by patients, but no conclusive scientific evidence exists on this issue[16,17]. In our series, the use of a Choostent device allowed optimal nutrition and tolerance of neo-adjuvant therapy until tumor downstaging was documented. The stent was easily removed under mild sedation within 2 mo in 7 patients, 3 of whom underwent surgical resection without complications.

In conclusion, covered nitinol stents are safe and effective devices for palliation of dysphagia in patients with esophageal strictures. The Ultraflex and the Choostent proved to be equally reliable in the achievement of this goal. Close patient monitoring is required to avoid late complications. When temporary stent insertion is required, as in patients undergoing neo-adjuvant therapy and in those with anastomotic complications or post-radiotherapy strictures, the Choostent is preferable because of its easy and safe removal.

COMMENTS

Background

Less than 50% of patients with esophageal carcinoma are suitable for surgery at the time of diagnosis. In such circumstances, the self-expanding metal stents (SEMS) represent an excellent palliative option. Covered SEMS have also been used in the management of patients with anastomotic complications, malignant extrinsic compression of the esophagus, and post-radiotherapy stricture.

Research frontiers

Despite the large number of covered metal stents available on the market, the superiority of one type over another has not been proven yet in the management of esophageal strictures and leaks.

Innovations and breakthroughs

This study compared two different types of nitinol stents that were proven to be equally reliable for palliation of dysphagia and leaks. Both stents also proved to be easily removable up to 2 mo after insertion.

Applications

Temporary stent placement has a role in patients undergoing neo-adjuvant therapy and in those with anastomotic complications or post-radiotherapy strictures. This minimally invasive and reversible treatment can represent an alternative to trans-nasal feeding tube placement, endoscopic percutaneous gastrostomy, or jejunostomy in selected patients.

Peer review

This is an interesting and worthwhile report on the use of expandable covered metal stents for the treatment of esophageal problems.

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BRIEF ARTICLE

Insulin resistance is associated with hepatocellular carcinoma in chronic hepatitis C infection

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Abstract

AIM: To elucidate the role of insulin resistance (IR) and serum adiponectin level in hepatocellular carcinoma (HCC) associated with chronic hepatitis C.

METHODS: Clinical and biochemical characteristics were collected from 165 consecutive patients with newly diagnosed HCC. Homeostasis model assessment of IR (HOMA-IR) and serum adiponectin level were investigated in 188 patients with different stages of hepatitis C virus (HCV) infection.

RESULTS: Among HCC patients, type 2 diabetics (DM) was more prevalent in HCV subjects (35.6%, n = 59) compared to hepatitis B virus (HBV; 12.7%, n = 63) or non-HBV, non-HCV cases (7.1%, n = 28). In patients with chronic hepatitis C, HCC subjects had higher blood sugar (P < 0.001), insulin level (P = 0.003) and HOMA-IR (P = 0.018) than those with chronic hepatitis and advanced fibrosis. Age, male sex and body mass index were significantly associated with serum adiponectin level, whereas HOMA-IR was not. Based on stepwise logistic regression analysis, age (OR: 1.124, P < 0.001), serum insulin level (OR: 1.585, P < 0.001), HOMA-IR (OR: 0.495, P = 0.001), DM (OR: 11.601, P = 0.002) and male sex (OR: 3.877, P = 0.016) were independently associated with HCC. This result was similar even if the diabetic subjects were excluded for analysis.

CONCLUSION: Insulin resistance measured by HOMA-IR, regardless of the presence of diabetes, is significantly associated with HCC development in patients with chronic HCV infection.

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Key words: Hepatitis C virus; Hepatocellular carcinoma; Insulin resistance; Diabetes; Adiponectin

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INTRODUCTION

Hepatitis C virus (HCV) infects hundreds of millions of

people persistently, and induces a spectrum of chronic liver disease worldwide[1]. Chronic HCV infection causes progressive hepatic fibrosis and cirrhosis in up to 20% of patients, and approximately 10%-20% of cirrhotic patients may develop hepatocellular carcinoma (HCC) within 5 years[2-4]. Recent cohort studies have indicated that HCC is the most frequent cause of death in patients infected with HCV, and epidemiological trends suggest that the mortality rate is rising[5]. Thus, understanding the risk factors for HCC development in patients infected with HCV is of great importance.

HCV may contribute to carcinogenesis by causing advanced fibrosis or cirrhosis, which represents a precancerous state accompanied by increased DNA synthesis[6,7]. Nevertheless, several factors associated with HCC development in chronic hepatitis C have been reported, such as male sex, older age at infection, excessive alcohol consumption, coinfection with hepatitis B virus (HBV) and some viral variability in HCV itself[8-11]. Recently, epidemiological studies have demonstrated that diabetes mellitus (DM) is associated with a 2-4-fold increase in the risk of HCC, regardless of the presence of other major HCC risk factors (HBV, HCV, and alcoholic liver disease)[12-15]. In particular, two large cohort studies have shown that DM is associated with a higher risk of HCC development in patients with chronic hepatitis C compared to HBV-infected subjects or those without HBV and HCV infections[12,13].

The mechanisms that may link DM with carcinogenesis in chronic HCV infection remain unclear. Insulin resistance (IR), which correlates inversely with circulating adiponectin concentration, is a consistent finding in patients with type 2 DM[16,17]. Previous studies have shown that patients infected with HCV have significantly higher IR than healthy controls matched for age, sex and body mass index (BMI)[18,19]. Recent studies have suggested that IR plays a crucial role in fibrosis progression, and has been demonstrated to have a negative impact on treatment responses to antiviral therapy in patients with chronic hepatitis C[18,20,21]. IR has emerged as a risk factor for a wide variety of cancers, including endometrial and breast (especially after menopause), colon and rectal, esophageal, kidney, pancreatic, biliary, ovarian and cervical cancer[22-24]. To the best of our knowledge, the role of IR and serum adiponectin level in the development of HCC associated with chronic HCV infection has not been established. In this present study, we prospectively investigated the IR assessed by the homeostasis model (HOMA-IR) and serum adiponectin level in patents with HCC and those with other clinical stages of chronic HCV infection.

MATERIALS AND METHODS

Patients

From January 2007 to August 2008, a total of 165 newly diagnosed patients with HCC (122 men and 43 women; median age: 60.1 ± 12.4 years) who fulfilled all criteria outlined below were consecutively collected in a single center. The diagnosis of HCC was based on either the histopathological findings in tumor tissue, one typical HCC feature on a dynamic image or alpha-fetoprotein (AFP) > 200 ng/mL if the nodule was > 2 cm in cirrhotic liver, or two typical HCC features of dynamic images if the nodule was between 1 and 2 cm in a cirrhotic liver[25]. Patients with concurrent human immunodeficiency virus infection, significant change of body weight (≥ 3 kg within 3 mo), previous history of interferon-based antiviral therapy, and current treatment with any dosage of insulin therapy were excluded.

Of these patients, 63 were positive for hepatitis B surface antigen (HBsAg) (Abbott Laboratories, North Chicago, IL, USA), 59 were positive for anti-HCV antibody (third-generation ELISA; AxSYM HCV 3.0; Abbott Laboratories), 15 were positive for both HBsAg and anti-HCV, and 28 were negative for HBsAg and anti-HCV, in whom alcoholic liver disease (n = 11, 39%) was the major cause of HCC.

During the same period, 129 consecutive patients (61 men and 68 women, 23-77 years old; median age: 53.0 ± 11.5 years) with chronic HCV infection who consulted our clinics were studied, including 86 with chronic hepatitis (F0-2) and 43 with advanced fibrosis (F3-4). All patients had positive anti-HCV antibody and detectable HCV RNA (Amplicor™; Roche Diagnostics, Branchburg, NJ, USA). Pathological diagnosis of chronic hepatitis or advanced fibrosis was made by percutaneous liver biopsies according to the modified Knodell histological activity index[26], which were analyzed by pathologists who were blind to the patients' characteristics. The Human Research and Ethics Committee (Institutional Review Board) approved the study, and informed consent was obtained from each patient involved in the study.

Clinical and laboratory assessments

Patients with a BMI of 18.5-24.9 kg/m^2 were classified as normal, 25-29.9 as overweight, and ≥ 30 as obese. The diagnosis of type 2 DM was based on the American Diabetes Association revised criteria, using a value of fasting blood glucose of ≥ 126 mg/dL on at least two occasions[27], or ongoing treatment with hypoglycemic agents.

Blood glucose, serum insulin level and stored serum samples for adiponectin were collected after 12 h of overnight fasting from each individual. For HCC patients, serum samples were collected before any treatment for tumor. Serum insulin was measured by radioimmunoassay (Coat-A-Count insulin kit; Diagnostic Products Corp., Los Angeles, CA, USA). IR was calculated by the HOMA-IR using the following formula: HOMA-IR = fasting insulin (μU/mL) × plasma glucose (mmol/L)/22.5. Circulating level of adiponectin was measured in duplicate by sandwich ELISA using commercial kits according to the manufacturer's instructions (Quantikine ELISA kits; R&D Systems, Inc., Minneapolis, MN, USA). The differences between duplicate wells were consistently less than 10% of the mean values. The mean values of duplicate measurements were used in the analyses.

Table 1 Comparison of baseline features among HBV, HCV, dual HBV and HCV, and non-HBV, non-HCV related hepatocellular carcinoma patients

	HBV *n* = 63	HCV *n* = 59	HBV + HCV *n* = 15	Non-HBV, non-HCV *n* = 28	*P* value
Age (yr)[1]	55 (46-66)[a,c]	66 (57-75)[a,d]	60 (50-68)[d]	61 (55-67)[c]	< 0.001
Sex (M/F)	45/18	41/18	10/5	26/2	0.093
DM (%)	8 (12.7)[a]	21 (35.6)[a,e]	4 (26.7)	2 (7.1)[e]	0.003
BMI (kg/m^2)[1]	24.3 (22.2-26.8)	23.8 (21.8-26.6)	23.6 (20.9-25.5)	24.5 (23.3-26.7)	0.439
Overweight (%)	24 (38)	22 (37)	4 (27)	10 (36)	0.797
Obesity (%)	4 (6)	5 (8)	1 (7)	2 (7)	0.965
AST (U/L)[1]	48 (38-71)[a]	72 (39-123)[a,e]	72 (47-111)	38 (29-52)[e]	0.002
ALT (U/L)[1]	45 (34-63)[a]	63 (29-100)[a,e]	82 (54-117)	27 (21-39)[e]	0.011
Child–Pugh class (A/B, C)	47/16	46/13	11/4	23/5	0.857
Platelet (10^4/μL)[1]	16.5 (12.0-25.0)[a]	13.2 (8.7-17.1)[a,e]	12.7 (7.6-18.1)[f]	21.1 (15.3-24.1)[e,f]	0.001
AFP (ng/mL)[1]	15 (6-525)	28 (7-256)	38 (11-4388)	15 (6-8027)	0.468
Tumor size (≤ 3 cm) (%)	14 (22.2)[a]	29 (49.2)[a,e]	7 (46.7)	5 (17.9)[e]	< 0.001
BCLC (≤ early stage) (%)	20 (31.7)[a,b]	31 (52.5)[a,e,f]	9 (60.0)[b]	4 (14.3)[e,f]	0.001
HOMA-IR	3.5 (2.0-8.6)[a]	4.4 (2.9-6.6)[a]	3.2 (1.7-10.7)	3.4 (2.0-4.6)	0.108
Insulin (μU/mL)[1]	12.8 (8.4-25.0)	14.8 (9.9-21.30)	11.9 (8.1-23.5)	9.7 (7.6-14.8)	0.256
Adiponectin (μg/mL)	4.7 (2.9-8.1)[a,c]	7.9 (5.2-11.0)[a,e]	8.0 (3.8-10.9)[f]	3.7 (2.0-6.0)[c,e,f]	0.002

[1]Median (interquartile range); *P* value by one-way ANOVA test or χ^2 test; [a]$P < 0.05$ between HBV and HCV; [b]$P < 0.05$ between HBV and HBV + HCV; [c]$P < 0.05$ between HBV and non-HBV, non-HCV; [d]$P < 0.05$ between HCV and HBV + HCV; [e]$P < 0.05$ between HCV and non-HBV, non-HCV; [f]$P < 0.05$ between HBV + HCV and non-HBV, non-HCV HCC with LSD post-hoc correction. HBV: Hepatitis B virus; HCV: Hepatitis C virus; DM: Diabetes mellitus; BMI: Body mass index; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; AFP: Alpha-fetoprotein; BCLC: Barcelona clinic liver cancer; HOMA-IR: Homeostasis model assessment of insulin resistance.

Statistical analysis

Continuous data are expressed as the median (interquartile range), and the categorical data are expressed as a number (percentage). Comparisons of differences in the categorical date between groups were performed using the χ^2 test. Distributions of continuous variables were analyzed by the Mann-Whitney *U* test or one-way ANOVA test with least significant difference (LSD) post-hoc correction between groups where appropriate. Spearman's correlation coefficient analysis was used to evaluate the factors associated with HOMA-IR and adiponectin level. Multiple linear regression analysis with stepwise variable selection was performed to assess the independent factors. Stepwise logistic regression analysis was performed to assess the influence of each factor on the risk of developing HCC. All analyses were carried out using SPSS software version 15.0 (SPSS Inc., Chicago, IL, USA). All tests were two-tailed, and $P < 0.05$ was considered statistically significant.

RESULTS

Comparison of baseline features among HBV, HCV, dual HBV/HCV, and non-HBV, non-HCV-related HCC

Table 1 shows the comparison of baseline features among the 165 patients with HCC related to different etiology. The median age of HCV-related HCC patients (66 years) was significantly higher than that in HCC patients infected with HBV (55 years) or dual HBV/HCV (60 years) ($P < 0.001$). There was a male predominance among all four groups. The prevalence of DM was higher (35.6%) in patients with HCV-related HCC compared to those infected with HBV (12.7%) ($P < 0.005$) or non-HBV, non-HCV subjects (7.1%) ($P < 0.005$). HOMA-IR was higher in HCC patients with HCV (median 4.4) than in those with HBV (median 3.5) ($P < 0.05$). However, there was no significant difference in BMI and prevalence of overweight and obesity among the four groups. Patients with HCV-related HCC had significantly higher aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels and lower platelet counts than those infected with HBV or non-HBV, non-HCV subjects. In addition, patients with HCV-related HCC had smaller tumors and earlier Barcelona clinic liver cancer stage (BCLC) than HBV or non-HBV, non-HCV subjects.

Comparison of baseline features, serum insulin, HOMA-IR and adiponectin level among chronic hepatitis, advanced fibrosis and HCC patients

The comparison of baseline features, serum insulin, HOMA-IR and adiponectin level in different clinical stages of chronic HCV infection is shown in Table 2. The median age for HCC patients (66 years) was significantly higher than those with advanced fibrosis (56 years) and chronic hepatitis (53 years) ($P < 0.001$). Patients with HCC had a higher male-to-female ratio and higher prevalence of DM than those with advanced fibrosis or chronic hepatitis. There was no significant difference in BMI among these three groups. The HCC subjects had lower AST and ALT levels compared to those with advanced fibrosis; however, the platelet count was comparable between these two groups. Patients with HCC had higher blood sugar ($P < 0.001$), insulin level ($P = 0.003$) and HOMA-IR ($P = 0.018$) than those with chronic hepatitis and advanced fibrosis. As shown in Figure 1, patients with HCC had a higher ratio of HOMA-IR > 4 (61.8%, 95% CI: 48.6%-75.1%) than those with chronic hepatitis (39.5%, 95% CI: 29.0%-50.1%) and advanced fibrosis (48.8%, 95% CI: 33.3%-64.4%) ($P = 0.036$). There was no significant difference in serum adiponectin among the three groups.

Table 2 Comparison of baseline features, HOMA-IR and adiponectin level among chronic hepatitis, advanced fibrosis and HCC patients with chronic HCV infection

	Chronic hepatitis (F0-2) *n* = 86	Advanced fibrosis (F3-4) *n* = 43	Hepatocellular carcinoma *n* = 59	*P* value[2]
Age (yr)[1]	53 (45-58)[a,b]	56 (49-63)[a,c]	66 (57-75)[b,c]	< 0.001
Male sex (%)	44 (51)[b]	17 (40)[c]	41 (69)[b,c]	0.008
DM (%)	13 (15)[b]	12 (28)	21 (36)[b]	0.016
BMI (kg/m^2)[1]	24.3 (21.8-26.3)	25.0 (22.0-29.4)	23.8 (21.8-26.6)	0.227
AST (U/L)[1]	90 (64-122)	114 (87-178)[c]	72 (39-123)[c]	0.085
ALT (U/L)[1]	127 (92-187)[b]	140 (102-199)[c]	63 (29-100)[b,c]	0.001
Platelet (10^4/μL)[1]	20.2 (15.8-22.4)[a,b]	13.7 (10.6-17.9)[a]	13.2 (8.7-17.1)[b]	< 0.001
Sugar (mg/dL)[1]	91 (87-101)[b]	98 (88-122)[c]	114 (94-172)[b,c]	< 0.001
Insulin (μU/mL)[1]	10.3 (7.7-14.4)[b]	11.7 (6.9-15.9)[c]	14.8 (9.9-21.3)[b,c]	0.003
HOMA-IR[1]	3.5 (2.6-4.7)[b]	4.1 (2.6-5.9)[c]	4.4 (2.9-6.6)[b,c]	0.018
Adiponectin (μg/mL)[1]	5.0 (3.4-8.4)	5.8 (4.1-9.7)	7.9 (5.2-11.0)	0.222

[1]Median (interquartile range); [2]*P* value by one-way ANOVA test or χ^2 test; [a]*P* < 0.05 between chronic hepatitis and advanced fibrosis; [b]*P* < 0.05 between chronic hepatitis and hepatocellular carcinoma; [c]*P* < 0.05 between advanced fibrosis and hepatocellular carcinoma with LSD post-hoc correction.

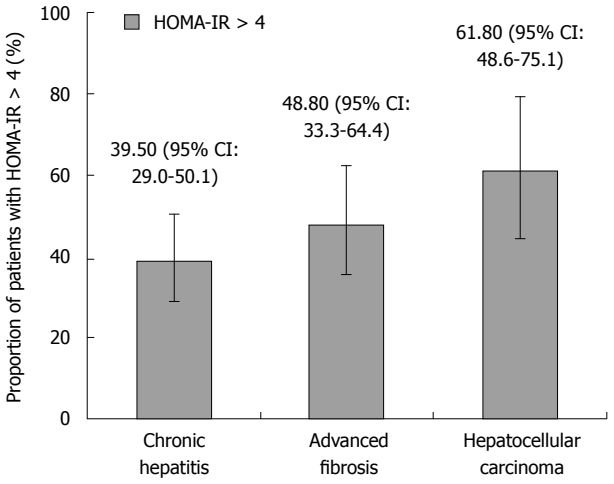


Figure 1 Comparison of high homeostasis model assessment of insulin resistance (HOMA-IR) (> 4) among different stages of chronic hepatitis C virus (HCV) infection (*P* = 0.036).

Factors associated with serum adiponectin level in patients with chronic hepatitis C

Table 3 shows the factors associated with serum adiponectin level in 188 patients with chronic HCV infection. By univariate analysis, age (r = 0.388, P < 0.001), male sex (P < 0.001), BMI (r = -0.281, P < 0.001), AST level (r = 0.159, P = 0.030), platelet count (r = -0.198, P = 0.009), insulin level (r = -0.179, P = 0.014) and HOMA-IR (r = -0.290, P < 0.001) were significant factors associated with serum adiponectin level. Multiple linear regression analysis showed that age (regression coefficient = 0.140, P < 0.001), male sex (regression coefficient = -2.925, P < 0.001) and BMI (regression coefficient = -0.495, P < 0.001) were independent variables.

Stepwise logistic regression analysis for factors associated with development of HCC

Based on stepwise logistic regression analysis, significant factors associated with development of HCC in patients with chronic HCV infection were age (OR: 1.124, 95% CI: 1.067-1.183, P < 0.001), serum insulin level (OR: 1.585, 95% CI: 1.269-1.980, P < 0.001), HOMA-IR (OR: 0.495, 95% CI: 0.330-0.743, P = 0.001), DM (OR: 11.601, 95% CI: 2.50-53.8, P = 0.002) and male sex (OR: 3.877, 95% CI: 1.282-11.729, P = 0.016) (Table 4).

When excluding DM cases, factors independently associated with HCC development in 142 non-DM patients were age (OR: 1.170, 95% CI: 1.075-1.272, P < 0.001), serum insulin level (OR: 2.434, 95% CI: 1.555-3.811, P < 0.001), HOMA-IR (OR: 0.158, 95% CI: 0.055-0.452, P = 0.001) and male sex (OR: 6.111, 95% CI: 1.310-28.49, P = 0.021).

Table 3 Univariate and multivariate analysis of factors associated serum adiponectin level in 188 patients with chronic HCV infection

	Univariate		Multivariate		
	Coefficient	*P* value[1]	Regression coefficient	SE	*P* value[3]
Age	0.388	< 0.001	0.140	0.028	< 0.001
Male sex[2]	NA	< 0.001	-2.925	0.744	< 0.001
BMI	-0.281	< 0.001	-0.495	0.101	< 0.001
HCC[2]	NA	0.136	-	-	-
DM[2]	NA	0.629	-	-	-
Child-Pugh classification	0.107	0.145	-	-	-
AST (U/L)	0.159	0.030	-	-	-
ALT (U/L)	-0.096	0.195	-	-	-
Platelet (10^4/μL)	-0.198	0.009	-	-	-
Insulin (μU/mL)	-0.179	0.014	-	-	-
HOMA-IR	-0.290	< 0.001	-	-	-

[1]*P* value by Spearman's test or [2]*P* value by Mann-Whitney *U* test; [3]*P* value by stepwise linear regression analysis. NA: Not applicable.

DISCUSSION

There is increasing evidence linking chronic HCV infection and type 2 DM. Large community-based studies have shown that the prevalence of DM in HCV-infected patients is much higher than that observed in the general population, and in patients with other chronic liver diseases such as HBV and alcoholic liver disease[28,29].

Table 4 Stepwise logistic regression analysis of factors associated with HCC

	Comparison	OR	95% CI	*P* value
All patients				
Age	Per 1 year increase	1.124	1.067-1.183	< 0.001
Insulin	Per 1 μU/mL increase	1.585	1.269-1.980	< 0.001
HOMA-IR	Per 1 increase	0.495	0.330-0.743	0.001
DM	Yes *vs* no	11.601	2.500-53.800	0.002
Sex	Male *vs* female	3.877	1.282-11.729	0.016
Non-DM patients				
Age	Per 1 year increase	1.170	1.075-1.272	< 0.001
Insulin	Per 1 μU/mL increase	2.434	1.555-3.811	< 0.001
HOMA-IR	Per 1 increase	0.158	0.055-0.452	0.001
Sex	Male *vs* female	6.111	1.310-28.499	0.021

In this study, we found that among HCC patients, type 2 DM was more prevalent in those infected with HCV compared to those with HBV or non-HBV, non-HCV infection. This observation in accordance with previous studies suggests a strong synergistic effect of metabolic factors and viral hepatitis in HCC development among HCV-infected patients[12,13]. Although there was no significant difference in BMI among HCC patients with different etiology, this could be explained by the low prevalence of obesity in our study population.

Chronic HCV infection is associated with the development of hepatic steatosis and unique, virus-specific alterations in host metabolism leading to the development of IR[19,30,31]. In this present study, we provide the first evidence that IR could potentially increase the risk of developing HCC in patients with chronic HCV infection. In a cross-sectional, hospital-based setting, we prospectively assessed the HOMA-IR value in different clinical stages of chronic HCV infection. Our data showed that patients with HCC had a higher ratio of HOMA-IR > 4 than those with chronic hepatitis and advanced fibrosis. Furthermore, after adjusting for age and sex, HOMA-IR was an independent factor associated with the development of HCC. A novel finding of our work, not specifically evaluated in other studies, was the association of IR, regardless of diabetes, with the development of HCC. An alternative explanation for the observed association between HOMA-IR and HCC is that advanced hepatic fibrosis and disease severity results in IR and impairs insulin clearance. This possibility could be excluded by the similar prevalence of DM and platelet count that has been considered as a fibrosis marker in chronic HCV infection between patients with HCC and those with advanced fibrosis. Also, HOMA-IR did not correlate with Child-Pugh classification, which suggests that disease severity was not associated with IR in patients with HCC or advanced fibrosis.

Although our work was not designed to clarify the pathogenic interaction between IR and the development of HCC, a few hypotheses can be put forward. IR is defined as an increased requirement for insulin to maintain normal metabolic function, which results in the compensatory development of hyperinsulinemia[32]. Recent evidence has suggested that hyperinsulinemia can promote the synthesis and biological activity of insulin-like growth factor 1 (IGF-1), which is a peptide hormone that regulates energy-dependent growth processes[33]. IGF-I stimulates cell proliferation and inhibits apoptosis and has been shown to have strong mitogenic effects on a wide variety of cancer cell lines. Changes in the expression pattern of IGF-system components have been observed in patients with HCC, in human HCC cell lines and in their conditioned culture medium, as well as in rodent models of hepatocarcinogenesis[34].

To study the role of adiponectin in HCC may be more complex because of its underlying chronic hepatitis infection[19,35,36]. Previous studies have demonstrated that circulating adiponectin levels are inversely associated with the risk of malignancies associated with IR, including endometrial, breast, colon and gastric cancer[37-40]. Moreover, serum adiponectin level has been reported to be significantly elevated in chronic liver disease, and correlated with stage of liver cirrhosis, liver cell injury, e.g. aminotransferase activity, and inflammatory markers[35,36]. Thus, serum adiponectin level is modified according to the two opposing factors, IR and underlying liver condition. In this study, we found no difference in serum adiponectin level among different clinical stages of chronic HCV infection. Although HOMA-IR score was inversely associated with serum adiponectin level by univariate analysis, multiple linear regression analysis did not support this correlation.

There are some limitations to our study. First, the analysis was carried out in a cross-sectional setting with a relatively small number of HCC patients, and it would be interesting to determine whether this association holds true in longitudinal follow-up studies of larger groups of patients. Second, the cohort of patients, at low prevalence of obesity, was enrolled in a tertiary referral center for liver disease, which limits the broader application of the results. A further methodological issue resides in the inability to dissect the temporal relation between IR and HCC. Another limitation lies in the fact that there is some concern on the use of HOMA-IR in the presence of long-standing DM. However, a diagnosis of DM is *per se* expression of IR, and HOMA-IR is a less invasive, inexpensive, and less labor-intensive method to measure IR as compared with the glucose clamp test.

In conclusion, we demonstrated the independent association between IR and HCC development in chronic HCV infection. These findings may have important prognostic and therapeutic implications in the management of chronic HCV-infected patients. Since IR is a potentially modifiable factor, therapeutic intervention aimed at decreasing IR may be warranted in these patients.

COMMENTS

Background

Recent studies have demonstrated that diabetes mellitus (DM) is associated with high risk of hepatocellular carcinoma (HCC) in patients with chronic hepatitis C. Insulin resistance (IR), which correlates inversely with circulating adiponectin concentration, is a consistent finding in patients with type 2 DM. Chronic hepatitis C virus (HCV) infection has been reported to be associated with increased IR. Recent studies have suggested that IR plays a crucial role in

fibrosis progression, and has been demonstrated to have a negative impact on treatment responses to antiviral therapy in patients with chronic hepatitis C.

Research frontiers

IR has emerged as a risk factor for a wide variety of cancers. In a cross-sectional, hospital-based setting, the authors assessed the role of IR assessed by the homeostasis model (HOMA-IR) and serum adiponectin level in the development of HCC associated with chronic HCV infection.

Innovations and breakthroughs

The authors have provided the first evidence that IR can potentially increase the risk of developing HCC in patients with chronic HCV infection. A novel finding, not specifically evaluated in other studies, is the association of IR, regardless of diabetes, with the development of HCC.

Applications

These findings may have important prognostic and therapeutic implications in the management of chronic HCV-infected patients. Since IR is a potentially modifiable factor, therapeutic intervention aimed at decreasing IR may be warranted in these patients.

Peer review

The authors present a clinical investigation of the correlation between IR and HCC. The title accurately reflects the major contents of the article, and the abstract delineates briefly and concisely the research background, objectives, materials and methods, results and conclusions.

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BRIEF ARTICLE

Distribution of *gyrA* mutations in fluoroquinolone-resistant *Helicobacter pylori* strains

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Abstract

AIM: To investigate the resistance of *Helicobacter pylori* (*H. pylori*) to ciprofloxacin (CIP), levofloxacin (LVX) and moxifloxacin (MOX) in the Beijing area and to elucidate the resistance mechanisms.

METHODS: Seventy-nine *H. pylori* clinical strains, isolated from patients who had undergone upper gastrointestinal endoscopy in Peking University First Hospital from 2007 to 2009, were tested for their susceptibility to CIP, LVX and MOX using the *E*-test method. *H. pylori* strain 26695 was included in the susceptibility testing as a control strain. According to the minimal inhibitory concentration (MIC) values, a strain was classified as resistant to CIP, LVX or MOX when the MIC was > 1 μg/mL. We amplified by polymerase chain reaction (PCR) and sequenced the quinolone resistance-determining regions of the *gyrA* and *gyrB* genes from 29 quinolone-resistant and 16 quinolone-susceptible *H. pylori* strains selected at random.

RESULTS: In this study, the resistance rates of *H. pylori* to CIP, LVX or MOX were 55.7% (44/79), and the primary resistance rates were 26.6% (21/79). Patients with secondary resistance had received LVX in previous eradication treatments, but not MOX or CIP. Forty-five strains, including 29 CIP, LVX or MOX-resistant strains (MIC: 1.5-32 μg/mL) and 16 susceptible strains, were selected randomly from the 79 strains and used in PCR analysis. Among these 45 strains, 27 resistant strains had mutations in the *gyrA* gene, including 11 strains with mutations corresponding to Asp-91 (MIC: 2-32 μg/mL), one of which also had a mutation corresponding to Val-150, and 16 strains had mutations at Asn-87 (MIC: 4-32 μg/mL), three of which also had mutations corresponding to Arg-140 or Val-150. In addition, Arg-140, Val-150 or Ala-97 mutations were separately detected in three susceptible strains. Analysis of the *gyrB* gene showed that one strain of low resistance had a mutation corresponding to Ser-457 that coexisted with an Asp-91 mutation. There was a significant difference in the occurrence of mutations in the *gyrA* gene between CIP, LVX and MOX-resistant and -susceptible strains ($P < 0.05$), but 2 resistant strains were found to possess no quinolone resistance-determining region mutations.

CONCLUSION: Resistance is primarily mediated through point mutations in gyrA. Whether other mechanisms are responsible for resistance in strains without mutations in the QRDR should be detected.

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Key words: *Helicobacter pylori*; Antibiotic resistance; Quinolones; Mutation; *gyrA*

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INTRODUCTION

Helicobacter pylori (*H. pylori*) infection, which affects half of the world's population, is responsible for gastritis[1], peptic ulcers[2,3] and gastric mucosa-associated lymphoid tissue lymphoma[4], and is a major risk factor for the development of gastric adenocarcinoma[5]. Eradication therapy plays an important role in the treatment of *H. pylori* infection. According to the Maastricht Ⅲ and Chinese Consensus Report, triple therapy using a proton-pump inhibitor (PPI) combined with clarithromycin and amoxicillin or metronidazole, is recommended as the first choice treatment[6,7]. With the increasing frequency of clarithromycin-resistance among *H. pylori* strains, there is rising concern about the potential decline in the eradication rate of this infection[8,9]. There is therefore an urgent need to introduce other treatment options. Fluoroquinolones, such as levofloxacin (LVX) and moxifloxacin (MOX), have been evaluated as an alternative to standard antibiotics against *H. pylori*[6,7].

Some studies have shown good results when using fluoroquinolone-based triple therapies for *H. pylori* eradication. In a German study, a 7-d course including LVX in patients with persistent *H. pylori* infection, resulted in eradication rates of greater than 85%[10]. In an Italian study, *H. pylori* eradication was achieved in 90% of patients treated with MOX, clarithromycin and lansoprazole[11]. However, the widespread use of fluoroquinolones for the treatment of *H. pylori* infection has led to an increase in its resistance rate in some areas, leading to unacceptably low eradication rates[12]. Several studies have shown that LVX-based therapies are not superior to traditional quadruple therapy or Maastricht triple therapy in the treatment of *H. pylori* infection, especially in the case of resistant *H. pylori* strains[13,14]. A Turkish study speculated that the low eradication rate with MOX-containing treatment regimens may be due to the development of resistance to this quinolone[15]. The findings from all of these studies indicate that a regimen that is effective in one area may not be effective in another area, as antibiotic-resistant rates for *H. pylori* may be different in different areas.

The fluoroquinolone resistance rates of *H. pylori* have been reported for several regions, including China (Mainland), Hong Kong, Taiwan, France, Japan and Korea[12,16-20], and range from 3% to about 20%. It was reported that LVX resistance was associated with prior fluoroquinolone use over the previous 10 years, and with the total number of fluoroquinolone courses prescribed[21]. A study from Korea speculated that resistance to MOX might be acquired through prior use of fluoroquinolones due to the development of cross-resistance to other fluoroquinolones like ciprofloxacin (CIP) and LVX[12]. Bogaerts *et al*[22] reported that mutations in the *gyrA* gene elevated the minimal inhibitory concentrations (MICs) to LVX, CIP and MOX.

The mechanism of action of fluoroquinolones is *via* inhibition of DNA gyrase and topoisomerase, which control and modify the topological state of DNA in cells. Fluoroquinolone then interferes with bacterial DNA replication. Both DNA gyrase and topoisomerase are composed of two A and two B subunits, encoded by the *gyrA* and *gyrB* genes for DNA gyrase and the *parC* and *parE* genes for topoisomerase Ⅳ. The mechanism of fluoroquinolone resistance in *H. pylori* has been found to be linked to mutations in the quinolone resistance-determining regions (QRDRs) of the *gyrA* gene. Mutations in the *gyrB* gene have also been reported in LVX-resistant strains isolated in Japan, but these often occurred along with *gyrA* mutations[19]. The resistance of *H. pylori* strains to fluoroquinolones in the Beijing area has not yet been reported. The aim of this study was to assess the prevalence of fluoroquinolone resistance against CIP, LVX and MOX in *H. pylori* strains isolated from patients over the past 3 years in Beijing and to compare the susceptibility of these strains with CIP and two newer fluoroquinolones *in vitro*. Mutations in the QRDRs of the *gyrA* and *gyrB* genes were also evaluated in these strains.

MATERIALS AND METHODS

Patients and bacterial strains

Seventy-nine clinical *H. pylori* strains were collected from adult patients, who were randomly selected and had undergone a gastroduodenoscopy at Peking University First Hospital between January 2007 and July 2009. Some patients had received *H. pylori* eradication therapy before, but none of the patients had received MOX or CIP-based therapy. The biopsy specimens were cultured on Colombia blood agar (BBL Microbiology Systems, Cockeysville, MD, USA), supplemented with 8% defibrinated sheep blood, and incubated for 5-7 d under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2). Clinical isolates were identified as *H. pylori* based on positive tests for urease, oxidase and catalase. All cultures were stored at -80℃ in brain-heart infusion broth (BHI, Difco Laboratory, Detroit, MI, USA) supplemented with 30% glycerol.

MIC determination

The MICs of LVX, CIP and MOX were determined by *E*-test strips (AB Biodisk, Solna, Sweden) according to the recommendations of the Clinical and Laboratory Standards Institute (Pennsylvania, USA) and the manufacturer's instructions. *H. pylori* 26695 was used as the quality control strain. The resistance breakpoints of fluoroquinolones were defined as > 1 μg/mL, as previously suggested[19,20].

Polymerase chain reaction amplification and nucleotide sequence analysis

H. pylori genomic DNA was extracted by the High Pure

polymerase chain reaction (PCR) Template Preparation kit (Tiangen, Beijing China). Oligonucleotide primers gyr APF (5'-AGCTTATTCCATGAGCGTGA-3') and gyr APR (5'-TCAGGCCCTTTGACAAATTC-3'), gyrBPF (5'-CCCTAACGAAGCCAAAATCA-3') and gyr BPR (5'-GGGCGCAAATAACGATAGAA-3') were designed to amplify a 582-bp and 465-bp region of the *H. pylori gyrA* and *gyrB* genes respectively. Primers were synthesized by Shenggong (Shanghai, China). PCR was performed in a 25 μL reaction volume containing 2 pmol of the oligonucleotide primer, 200 μmol/L (each) of dATP, dCTP, dGTP and dTTP (GE Healthcare, Little Chalfont, UK), 1.5 mmol/L of reaction buffer (GE Healthcare), 1 μL of template DNA, and 2.5 U of *Taq* polymerase (GE Healthcare). Thermocycling conditions were 94℃ for 5 min, followed by 35 cycles of 94℃ for 30 s, 53℃ for 30 s and 72℃ for 30 s, with a final extension step of 72℃ for 10 min. The reaction products were visualized by running 5 μL of the reaction mixture on 1.5% agarose gels. Sequencing of the amplified DNA was performed on an ABI 3730xl sequencer (Applied Biosystems, Foster City, CA, USA). The sequences were then compared with the published sequence of the *H. pylori gyrA* and *gyrB* gene (GenBank accession number NC_000915.1).

Statistical analysis

The association between MIC levels and the occurrence of *gyrA/B* mutations relating to quinolone susceptibility was determined using Fisher's exact probability test, a P-value < 0.05 was considered significant.

RESULTS

Antimicrobial susceptibility

The MICs of CIP, LVX and MOX were determined for the 79 *H. pylori* strains isolated between 2007 and 2009 from gastric biopsies. The resistance rate to either CIP, LVX or MOX was 55.7% (44/79). Primary CIP, LVX and MOX resistance was detected in 21 (26.6%) isolates. Based on the breakpoints, we found that the strains susceptible to CIP (MIC ⩽ 1.0 μg/mL) were also susceptible to LVX and MOX, whereas the isolates resistant to CIP (MIC > 1.0 μg/mL) were also resistant to LVX and MOX (Tables 1 and 2).

Mutation analyses of the gyrA and gyrB genes and the association with LVX, MOX and CIP resistance

Of the 45 strains selected randomly, 29 were resistant and 16 were susceptible to LVX, MOX and CIP. Various substitutions in the QRDR of the *gyrA* and *gyrB* genes were observed in 31 of the strains. Mutations in the *gyrA* gene were identified in 27 resistant strains, 11 of which had mutations corresponding to Asp-91 and 16 of which had mutations corresponding to Asn-87. As for the *gyrB* gene, one low-level resistant strain had a mutation corresponding to Ser-457 which coexisted with the Asp-91 mutation. One susceptible strain exhibited a mutation corresponding to Val-451 of the GyrB protein. Amino acid substitutions in the GyrA protein associated with quinolone resistance fell into the following categories: (1) substitution of the amino acid at position 91 in 10 of the 27 isolates; (2) substitution of the amino acid at position 87 in 13 isolates; and (3) two mutations leading to substitution of amino acids in four isolates (Table 1). We also found 2 resistant strains with no QRDR mutations; in these strains the MICs to fluoroquinolones were > 12 μg/mL (Table 2).

Table 1 MICs and amino acid changes in quinolone-resistant and susceptible strains of *H. pylori*

Strains	MIC (μg/mL)			Mutations of *GyrA*	Mutations of *GyrB*
	LVX	MOX	CIP		
1R[1]	1.5	2	32	Asp91Gly and Val150Ala	0
2R[1]	2	2	32	Asp91Asn	0
3R[1]	2	2	32	Asp91Gly	0
4R[1]	3	1.5	1.5	Asp91Asn	Ser457Ala
5R[1]	4	3	4	Asp91Tyr	0
6R	4	3	32	Asp91Asn	0
7R[1]	6	4	32	Asp91Asn	0
8R	6	6	12	Asn87Lys and Arg140Lys	0
9R	8	4	12	Asn87Tyr	0
10R	12	8	32	Asp91Gly	0
11R	12	12	12	Asn87Ile and Arg140Lys	0
12R[1]	12	12	32	Asp91Asn	0
13R	32	12	32	Asn87Tyr	0
14R[1]	32	16	32	Asp91Asn	0
15R[1]	32	32	32	Asp91Asn	0
16R[1]	32	32	32	Asn87Ile	0
17R	32	32	32	Asn87Ile and Val150Ala	0
18R[1]	32	32	32	Asn87Lys	0
19R[1]	32	32	32	Asn87Lys	0
20R	32	32	32	Asn87Lys	0
21R	32	32	32	Asn87Lys	0
22R	32	32	32	Asn87Lys	0
23R	32	16	8	Asn87Lys	0
24R	24	32	32	Asn87Lys	0
25R	12	12	32	Asn87Lys	0
26R	6	6	32	Asn87Lys	0
27R	3	6	12	Asn87Lys	0
1S	0.02	0.02	0.016	Val150Ala	0
2S	0.016	0.016	0.032	Ala97Thr	0
3S	0.064	0.094	0.064	Arg140Lys	0
4S	0.064	0.02	0.023	0	Val451Gly

[1]Primary resistance. R: Resistant strains; S: Susceptible strains; 0: No mutation; MIC: Minimal inhibitory concentration; LVX: Levofloxacin; MOX: Moxifloxacin; CIP: Ciprofloxacin; *H. pylori*: *Helicobacter pylori*.

Table 2 MICs and amino acid changes in quinolone strains of *H. pylori* with no QRDR mutations

Strains	MIC (μg/mL)			Mutations of *GyrA*	Mutations of *GyrB*
	LVX	MOX	CIP		
28R[1]	24	16	32	0	0
29R[1]	16	32	32	0	0

[1]Primary resistance. QRDR: Quinolone resistance-determining region.

DISCUSSION

In this study, we investigated the resistance of *H. pylori* strains isolated from patients at Peking University First Hospital during 2007-2009 to three quinolone antibiotics. Of the 79 *H. pylori* strains isolated, 44 (55.7%) strains

were found to be resistant to quinolones and 21 (26.6%) showed primary resistance to quinolones. This is the first report of primary *H. pylori* resistance to quinolones in the Beijing area. In a previous study carried out in Hong Kong, the prevalence of LVX resistance in *H. pylori* was reported to be 11.5%[16]. An increase in fluoroquinolone resistance has also been reported in other areas, for example, the resistance rate to either CIP or LVX increased from 2.8% (1998-2003) to 11.8% (2004-2007) in southern Taiwan[17]. In other parts of the world, geographical differences and differences in treatment regimens result in a range of resistance rates, for example, rates of more than 20% in Korea[12], 15% in Japan[19] and 14% in Italy[23] have been reported.

According to research from Japan[19], caution is needed when interpreting these results because no criteria have been published for establishing the breakpoint of fluoroquinolones against *H. pylori*. According to the Maastricht Ⅲ Consensus Report[6], the threshold of clarithromycin resistance at which the empirical use of this antibiotic should be abandoned, or pretreatment clarithromycin susceptibility testing performed, is 15%-20%. Although some studies have reported good eradication rates using fluoroquinolones in some areas of China[24,25], quinolone treatment regimens in Beijing should be based on the findings of local antimicrobial susceptibility tests as the resistance rate is higher than 20% in this area.

In China, quinolone antibiotics have been widely used in hospitals to treat various infections and in the poultry industry as a supplement in feed. This has resulted in drug resistance becoming a serious problem[26-28]. In our study and a previous study from Korea[12], cross-resistance of the *H. pylori* strains to MOX, CIP and LVX was observed in patients who had not received MOX before the other eradication regimens.

Fluoroquinolone resistance is attributed to specific mutations in the genes encoding DNA gyrase and/or topoisomerase Ⅳ. In *Neisseria gonorrhoeae*[29], for example, mutations in the QRDRs of the *gyrA* and *parC* genes may be responsible for fluoroquinolone resistance. In *H. pylori*, only mutations in the DNA gyrase gene have been considered responsible for fluoroquinolone resistance because neither *parC* nor *parE* have been detected in the genome sequence and the drug efflux system is not considered important in this organism[30,31]. Consequently, DNA gyrase is the unique target for quinolones in *H. pylori*. Fluoroquinolone resistance of *H. pylori* is considered to depend on point mutations in the QRDR of the *gyrA/B* gene. GyrA mutations at Asn-87 and Asp-91 have been reported previously[19,22]. In our study, 11 resistant strains (37.93%) possessed mutations at Asp-91, 16 resistant strains (55.17%) possessed mutations at Asn-87, but mutations at both Asp-91 and Asn-87 were not found to coexist in any strain. Mutations at position Asn-87 were more frequent than at Asp-91 in LVX-resistant strains in Japan, according to a previous report [19]. We also identified more mutations at Asn-87 than at Asp-91 in this study. In *Escherichia coli*, Nakamura *et al*[32] found that mutations in the *gyrB* gene also lead to low-level quinolone resistance. Yoshida *et al*[33] identified two possible mutations in the GyrB protein of *Escherichia coli*: Asp426→Asn and Lys447→Glu. We also identified mutations in the *gyrB* gene in this study, but there was no statistically significant difference between the genotype of this gene and quinolone resistance. The role of GyrB in fluoroquinolone resistance therefore remains to be determined. In *H. pylori*, one mutation within the QRDR of the *gyrA* gene was associated with low level fluoroquinolone resistance, and two or more mutations may relate to high level of fluoroquinolone resistance[19]. One previous study reported that in *Campylobacter spp.*[34], a single mutation in the *gyrA* gene can lead to a high level of resistance not only to nalidixic acid but also to CIP, but it requires a second mutation to become MOX-resistant. In our study, two strains with two mutations in the QRDR of the *gyrA* gene had different levels of resistance to quinolones. Consistent with the findings of the Japanese study, we were also unable to identify any significant association between *gyrA* mutation patterns and the MICs of three fluoroquinolones[19]. In our study, we found that two resistant strains had no mutations in the QRDR of the *gyrA* and *gyrB* gene, and in these strains the MICs of three fluoroquinolone antibiotics reached 32 μg/mL. We also found that the same mutation can exist in resistant and susceptible strains. We therefore speculate that low-level resistance is most likely mediated through a point mutation in the *gyrA* gene. There may be other mutations in non-QRDRs of the *gyrA* and *gyrB* genes or other mechanisms, such as efflux systems, that lead to high level resistance.

A previous Japanese study reported that differences in amino acid substitutions associated with fluoroquinolone resistance correlate with geographical differences[19], with the occurrence of the Asn-87 GyrA mutation being more frequent than the Asp-91 GyrA mutation in LVX-resistant strains. Different from the findings of our study, it has previously been reported that in Hong Kong the most frequent mutation site in the GyrA protein was position 91[16].

In conclusion, the prevalence of resistance of *H. pylori* to fluoroquinolones is of concern in the Beijing area. Our results suggest that the susceptibility of *H. pylori* to fluoroquinolones should be tested before administration of a therapy, especially in the Beijing area. Resistance is most likely mediated through point mutations in the *gyrA* gene. Future studies should investigate whether other mechanisms are responsible for resistance in strains in which no mutations in the QRDR were detected.

COMMENTS

Background

Resistance to antibiotics is one of the most important reasons behind *Helicobacter pylori* (*H. pylori*) eradication failure. *H. pylori* resistance to metronidazole and clarithromycin is becoming an increasingly serious problem and as a result first-line treatment programs based on these drugs are declining. As an alternative to these standard regimens, quinolones can be recommended for the first-line treatment of *H. pylori* infection.

Research frontiers

Fluoroquinolone-based regimens for *H. pylori* eradication are widely employed

in some areas of China, but studies investigating fluoroquinolone resistance are limited.

Innovations and breakthroughs

Traditional *E*-tests and modern genetic mutation analysis of 45 *H. pylori* isolates showed a high rate of resistance to fluoroquinolones in Beijing. Resistance was found to be most likely mediated through point mutations in the *gyrA* gene, but two resistant strains were found with no quinolone resistance-determining region mutations. The possibility that other mechanisms are responsible for fluoroquinolone resistance in these strains remains to be determined.

Applications

The results of this study show a high prevalence of *H. pylori* resistance to fluoroquinolones in Beijing. The authors recommend that the susceptibility of *H. pylori* to fluoroquinolones is tested before the administration of a therapy.

Terminology

gyrA: The gene encoding DNA gyrase (type Ⅱ topoisomerase), subunit A; *gyrB*: The gene encoding DNA gyrase (type Ⅱ topoisomerase), subunit B.

Peer review

The manuscript investigates the prevalence and mechanisms of resistance of *H. pylori* to fluoroquinolones in Beijing. The methodology of resistance determination consists of a traditional *E*-test and modern genetic testing of *gyrA* mutation. The results show a primary resistance of 26% and secondary resistance of 55%, which is higher than in European countries. The article is good, using a sound methodology and correct conclusions.

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BRIEF ARTICLE

Most common *SLC25A13* mutation in 400 Chinese infants with intrahepatic cholestasis

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Abstract

AIM: To establish the real time fluorescence polymerase chain reaction (RT-PCR) with dual labeled probes for fast detection of *SLC25A13* gene mutation 851del4.

METHODS: Four hundred infants (< 1 year of age) with unexplained intrahepatic cholestasis from 18 provinces or municipalities in China were enrolled in this study for detecting their *SLC25A13* gene mutation 851del4. Suitable primers and fluorescence-labeled probes for detecting *SLC25A13* gene mutation 841del4 were designed. Normal and mutant sequences were detected by PCR with two fluorescence-labeled probes. After a single RT-PCR, results were obtained by analyzing the take-off curves. Twenty-four positive and 14 negative samples were retested by direct sequencing.

RESULTS: Eight homozygous and 30 heterozygous mutations were detected in 46 mutant alleles with a 851del4 mutation rate of 5.8% (46/800). Twenty-six and 20 mutant alleles were observed respectively, in 474 and 242 alleles from the intermediate and southern areas of China. No mutant allele was detected in 84 alleles from northern China. Twenty-four positive samples including 4 homozygous and 20 heterozygous mutations, and 14 negative samples were retested by direct sequencing, which confirmed that the accuracy of RT-PCR was 100%.

CONCLUSION: RT-PCR can detect the mutation 851del4 in infants with intrahepatic cholestasis with an accuracy of 100%.

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Key words: 851del4 mutation; Neonatal intrahepatic cholestasis; Real-time fluorescent polymerase chain reaction; *SLC25A13* gene

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Fu HY, Zhang SR, Yu H, Wang XH, Zhu QR, Wang JS. Most common *SLC25A13* mutation in 400 Chinese infants with intrahepatic cholestasis. *World J Gastroenterol* 2010; 16(18): 2278-2282 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i18/2278.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i18.2278

INTRODUCTION

The *SLC25A13* gene, localized in chromosome 7q21.3, encodes a 3.4kb transcript that is expressed most abundantly in liver[1,2]. *SLC25A13* gene mutations can lead to citrin deficiency, which causes not only onset of type II citrullinemia (CTLN2) in adults[2], but also neonatal intrahepatic cholestasis (NICCD)[3,4]. Clinical manifestations of NICCD

include intrahepatic cholestasis, fatty liver, failure to thrive, hyperaminoacidemia, liver dysfunction, hypoglycaemia, hypoproteinemia, and coagulation disorders. Symptoms of most NICCD patients disappear by 12 mo of age. Specific treatment, except for nutritional program including supplementation of fat soluble vitamins and formulas containing medium-chain triglycerides[3,5-7], is usually not required. However, NICCD is not always benign and a few NICCD patients may have a less favorable clinical course with relatively early liver dysfunction that necessitates management with liver transplantation under the age of 1 year[8]. Some infants with homozygous mutations may suffer from CTLN2 one or more decades later[9,10].

Since the full genomic sequence of the *SLC25A13* gene was published in 1999, over 50 mutations have been identified[11]. It has been reported that more than 100 000 East Asians have two homozygous mutated *SLC25A13* alleles, and the carrier rate in Chinese population is 1/63[11,12]. Mutation 851del4, is a 4-bp (GTAT) deletion from nt 851-854 in exon 9, leading to premature truncation of a protein. Population analysis has shown that mutation 851del4 accounts for 70% of all detected *SLC25A13* gene mutations in general Chinese population[13]. However, its prevalence in infants with intrahepatic cholestasis is unclear.

Traditionally, mutation 851del4 is tested by direct agarose gel electrophoresis or GeneScan[2,12-14]. Since the differences in length between the mutant fragment and wild sequence are quiet small, direct agarose gel electrophoresis depends greatly on experience of the investigator, while GeneScan requiring expensive equipments is time consuming. In this study, real-time fluorescence polymerase chain reaction (RT-PCR) with dual labeled probes was used to detect the most common *SLC25A13* gene mutation 851del4 in 400 Chinese infants with intrahepatic cholestasis.

MATERIALS AND METHODS

Study design

A gene test for mutation 851del4 in 14 infants with intrahepatic cholestasis was carried out by direct sequencing. Four cases were detected to be with mutant alleles, including one homozygote and three heterozygotes. These patients were used as samples to develop the method of RT-PCR. The other ten cases with wild sequence served as the control.

Then, RT-PCR was performed to detect the prevalence of 851del4 mutation in 386 infants with unexplained intrahepatic cholestasis. If a heterozygous mutation 851del4 was found in an infant, the second mutation was identified in all the 18 exons and their flanking sequences by direct sequencing.

The accuracy of RT-PCR was retested by direct sequencing in 24 infants with mutant sequences and 14 infants with normal sequence.

Subjects

From June 2003 to May 2009, 400 infants with idiopathic intrahepatic cholestasis were enrolled in this study for testing their genes. The inclusion criteria were conjugated hyperbilirubinaemia with its serum total bilirubin (TBil) exceeding 5 mg/dL and conjugated bilirubin level > 20% of TBil or > 1 mg/dL if the total serum bilirubin < 5 mg/dL, and development of conjugated hyperbilirubinaemia under the age of 1 year. Those were excluded from the study if they had diseases affecting their extrahepatic biliary system (such as biliary atresia, choledochal cyst or tumor, inspissated bile, or haemangioma), low γ-glutamyl transpeptidase (GGT) level (no more than 50 U/L) that may indicate progressive familial intrahepatic cholestasis[15], and obvious extrahepatic abnormalities or positive serology other than cytomegalovirus (CMV) that may also indicate infection. CMV infection occurs frequently in infants with neonatal hepatitis in Mainland China[16,17], and the outcome is similar in those infected with or without CMV[18,19].

The infants included in this study came from 18 provinces or municipalities in China. Because the most likely boundary between northern and southern China was drawn at an altitude of 30°N, the Yangtze River was considered a historically significant border between northern and southern China as previously described[13,20]. According to such criteria, 237 infants came from a border area (Shanghai City, and Jiangsu, Anhui, Hubei, Sichuan Provinces), 121 from the southern area (Hunan, Jiangxi, Zhejiang, Fujian and Guangdong Provinces), and 42 from the northern area (Jilin, Liaoning, Hebei, Shandong, Ningxia and Henan Provinces) in our study (Figure 1).

RT-PCR assay for mutation 851del4

This study was approved by the Ethics Committee on Human Research of the Children's Hospital of Fudan University. Informed consent was obtained from the parents or the guardians of each participant.

The sequence of upstream primer and downstream primer is 5'-TTGGTATATTTGTTGCTTGTGTTT-3' and 5'-AGAGGGAACTCTGCCCTTTAA-3', respectively. Their normal and mutated sequences were detected with the probe FAM-CCTACAGACGTATGACCTTAGCA-TAMRA and the probe HEX-CCTACAGACGACCTTAGCAGA-TAMRA, respectively. Twenty-five microlitre reaction mixture used in this study contained 2 μL of genomic DNA, 10 μL of 2.5* real Master Mix [Tiangen Biotech (Beijing) Co. Ltd.], 1.25 μL of 20*probe enhancer, 0.25 μL of each FAM- and HEX-labeled probe, 0.5 μL of each upstream primer and downstream primer (diluted to 10 μmol/L), and 10.25 μL of ddH_2O. After overlaying 25 μL liquid, RT-PCR was performed under the following conditions: denaturation at 95℃ for 2 min, followed by 40 thermal cycles, each at 95℃ for 15 s, at 56℃ for 30 s, and at 68℃ for 40 s. The results were obtained by analyzing the curves.

Conventional PCR assay for mutation 851del4

To ensure the specificity and sensitivity of amplification, nested PCR was performed to amplify exons 8 and 9. Thirty cycles of PCR were performed in 30 μL reaction mixture at

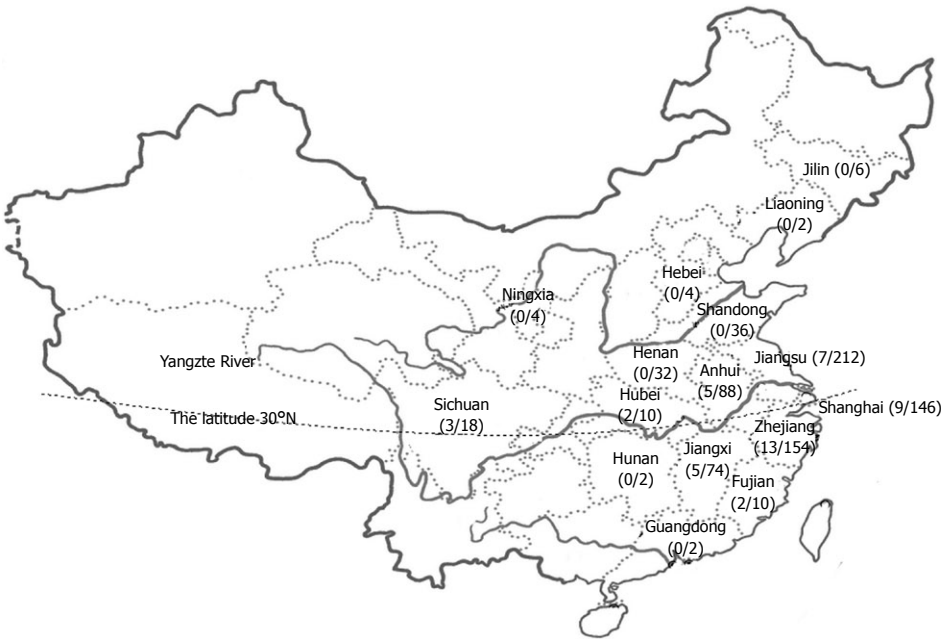


Figure 1 Distribution of idiopathic intrahepatic cholestasis infants enrolled in our study. As shown in the map, the provinces were separated into three parts by the Yangtze River and the northern latitude circle of 30°. The numbers in parentheses are the number of alleles of 851del4 mutation over the tested alleles.

94℃ for 3 min followed by 25 cycles, each at 94℃ for 30 s, at 50℃ for 30 s, at 72℃ for 1 min, and a final extension at 72℃ for 4 min, using primers Ex8/9F(5'-GCCTG-TAATCCCAGCACTT-3') and Ex8/9R(5'-GTTGAGA-GATGTAGCGAAAGAA-3'). The outer PCR reaction mixture consisted of 2*Taq- plus 15 μL PCR Master MIX, 1 μL in each primer (10 μmol/L), 2 μL DNA template, and 11 μL ddH_2O. After the initial outer PCR, inner PCR was conducted at 94℃ for 3 min followed by 30 cycles, each 94℃ for 30 s, at 50℃ for 30 s, at 72℃ for 1 min, and a final extension at 72℃ for 4 min using 1 μL of primers IVS7F (5'-TCACTCATTCCAGTGCCTTG-3') and IVS9B (5'-GCAGTTGCCTTTGCGGCATTG-3') as previously described[2], 2 μL outer PCR product as template, 2*Taq-plus 15 μL PCR Master MIX, and 11 μL ddH_2O.

The PCR products were analyzed by direct sequencing, in which IVS7B and IVS9B were taken as sequence primers for forward and backward sequencing identification. Mutation 851del4 was identified using software (Bioedit, North Carolina State University, NC, USA) and checked by two of the investigators.

RESULTS

Accuracy of RT-PCR

The curves were analyzed in accordance with the direct sequencing in 14 infants with known sequences. In infants with homozygous 851del4 mutation, positive fluorescence was observed only on the HEX-530 curve but not on FAM-490 curve. In those with heterozygous 851del4 mutation, positive fluorescence was found on both FAM-490 and HEX-530 curves, which was very close to each other, with the difference in *Ct* value always less than 2 (Figure 2). In those with the wild sequence, positive fluorescence was detected only on the FAM-490 curve. For any given sample, the experiment was considered a failure if negative fluorescence appeared on both HEX-530 and FAM-490 curves.

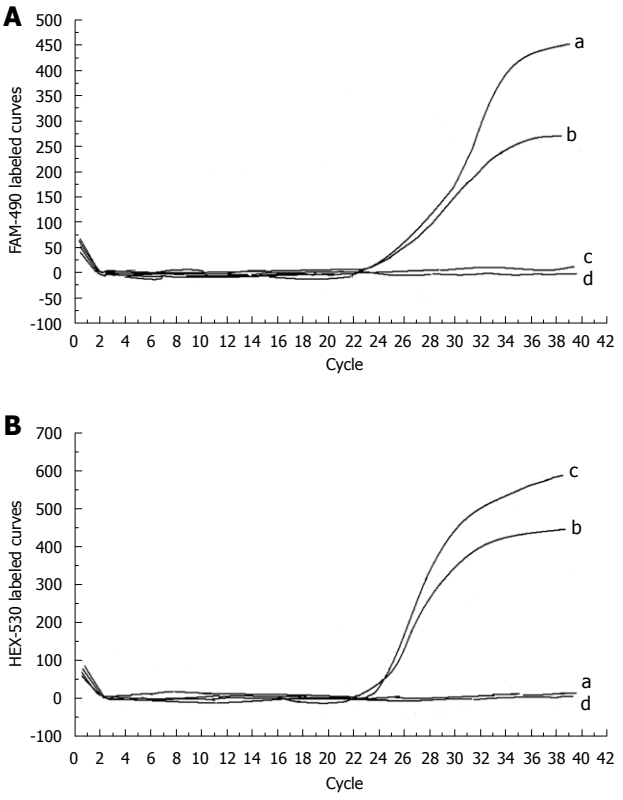


Figure 2 Positive fluorescences on FAM-labeled (A) and HEX-labeled (B) curves for different samples. As shown in the figure, positive fluorescence was observed on both FAM-labeled and HEX-labeled curves in sample b, indicating that b is a heterozygote. In sample c, positive fluorescence was only observed on the HEX-labeled curve, indicating that c is a homozygote. Positive fluorescence was observed on the FAM-labeled curve, otherwise HEX-labeled curve is a horizontal line in sample a, indicating that sample a has a wild sequence. d is a blank control with negative fluorescence on both FAM-labeled curve and HEX-labeled curve.

Positive mutation 851del4 was detected in 24 samples including 4 homozygous and 20 heterozygous mutations, and negative mutation 851del4 was retested in 14 infants

by nested PCR and direct sequencing, which was consistent with that detected by RT-PCR.

Prevalence of mutation 851del4

Eight homozygous and 30 heterozygous mutations were detected in the 400 infants with intrahepatic cholestasis, with a 851del4 mutation rate of 5.8% (46 mutant alleles with 851del4 were detected in 800 tested alleles). Subsequent testing of 18 exons and their flanking sequences in 30 heterozygous mutations showed another 9 mutant alleles including 1638ins23 in 5, R184X in 1 and novel mutations (data will be reported separately, manuscript in preparation) in 3, respectively.

Geographical variation of mutation 851del4 in China

Twenty-six and 20 mutant alleles were detected, respectively in 474 and 242 alleles of the intermediate and southern areas,with a 851del4 mutation rate of 5.5% and 8.3%, respectively. No mutant allele was detected in alleles of northern China (Figure 1).

DISCUSSION

To our knowledge, this is the first study to detect the most common mutation 851del4 of the *SLC25A13* gene in Chinese infants with intrahepatic cholestasis in a relatively large sample size. In this study, we developed a simple, fast, and accurate RT-PCR for detecting the mutation 851del4, the most common mutation of the *SLC25A13* gene, and detected 46 mutant alleles from 800 alleles with it. The prevalence of mutation 851del4 in Chinese infants with intrahepatic cholestasis was 1/17, which is much higher than that in general Chinese population (1/93)[13].

Compared with conventional nested PCR and agarose gel electrophoresis or GeneScan, RT-PCR could detect the mutation alleles and identify patients with homozygous or heterozygous 851del4 mutation, indicating that it can be used in diagnosis of intrahepatic cholestasis in infants.

In the present study, the mutation rate of 851del4 was about 5.8%, which was significantly higher than that in the general population, indicating that the *SLC25A13* gene mutation plays an important role in the pathogenesis of intrahepatic cholestasis of Chinese infants and should be detected in such infants, especially in those with symptoms of NICCD.

Screening of mutation 851del4 by RT-PCR may contribute to the diagnosis of intrahepatic cholestasis in patients with homozygous mutation because testing 18 exons of the *SLC25A13* gene can be omitted and diagnosis of citrin deficiency can be made. Early diagnosis and intervention of NICCD are extremely important[20] for the prevention of its serious consequences.

In this study, 30 heterozygous mutations of 851del4 were detected in 400 intrahepatic cholestasis infants with 9 compound heterozygous mutations detected by examining 18 exons of the *SLC25A13* gene. It is noteworthy that if suspicious patients were found to be heterozygous or when no mutation was found, all the 18 exons and their flanking sequence of the *SLC25A13* gene should be examined.

The *SLC25A13* gene mutation 851del4 has geographical variations in China and can be frequently found in southern China (1/70) but rarely in northern China (1/940)[13]. In this study, mutant alleles were detected in most infants from the intermediate and southern areas but not in those from northern China, showing that the distribution of the *SLC25A13* gene mutation 851del4 is different in south and north areas of China. Our finding is consistent with the reported data[13]. Thus, different methods should be used in dignosis of intrahepatic cholestasis in infants according to their geographical origin.

RT-PCR was established and the *SLC25A13* gene mutation 851del4 was detected in 400 infants using it in this study. However, it was used in detecting only one specific *SLC25A13* gene mutation, its prevalence might be underestimated. Further study is needed to highlight the importance of the *SLC25A13* gene mutations in infants with intrahepatic cholestasis and the application of RT-PCR in clinical practice.

In conclusion, RT-PCR can detect the most common *SLC25A13* gene mutation 851del4 in patients with intrahepatic cholestasis, thus providing a useful and effective tool for the diagnosis of NICCD.

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COMMENTS

Background

SLC25A13 gene mutations lead to citrin deficiency, which causes onset of type Ⅱ citrullinemia (CTLN2) in adults and neonatal intrahepatic cholestasis (NICCD). Up to date, over 50 mutations of *SLC25A13* have been identified, in which 851del4 is the most common mutation type. Traditionally, mutation 851del4 is screened by direct agarose gel electrophoresis or GeneScan. Since these methods are highly dependent on personal experience or need special equipments, a simple and accurate method for screening the mutation 851del4 is needed.

Research frontiers

Population analysis has shown that mutation 851del4 accounts for 70% of all mutations of the *SLC25A13* gene in general Chinese population. Mutation 851del4 is a 4-bp (GTAT) deletion from nt 851-854 in exon 9, leading to premature truncation of a protein. Early diagnosis of citrin deficiency is extremely important.

Innovations and breakthroughs

Real-time fluorescent polymerase chain reaction (RT-PCR) with dual-labeled probes was established, which could detect the mutation 851del4. The homozygosity, heterozygosity and wild sequence can be differentiated clearly. Compared with conventional nested PCR and agarose gel electrophoresis or GeneScan, RT-PCR does not require expertise in experimental operation.

Applications

The prevalence of mutation 851del4 in Chinese infants with intrahepatic cholestasis is 1/17, which is much higher than that in general Chinese population (1/93). RT-PCR may be used in diagnosis of intrahepatic cholestasis in infants.

Peer review

The authors obtained infants with intrahepatic cholestasis from various parts of China. Using RT-PCR with dual-labeled probes, they identified patients with homozygous or heterozygous 851del4 mutations and found that the mutation is more prevalent in southern than in northern part of China, indicating that RT-PCR can also be used in diagnosis of other genetic disorders in pediatric patients.

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BRIEF ARTICLE

Adhesion and immunomodulatory effects of *Bifidobacterium lactis* HN019 on intestinal epithelial cells INT-407

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Abstract

AIM: To elucidate the adherence and immunomodulatory properties of a probiotic strain *Bifidobacterium lactis* (*B. lactis*) HN019.

METHODS: Adhesion assays of *B. lactis* HN019 and *Salmonella typhimurium* (*S. typhimurium*) ATCC 14028 to INT-407 cells were carried out by detecting copies of species-specific genes with real-time polymerase chain reaction. Morphological study was further conducted by transmission electron microscopy. *Interleukin-1β* (*IL-1β*), *interleukin-8*, and *tumor necrosis factor-α* (*TNF-α*) gene expression were assessed while enzyme linked immunosorbent assay was used to detect IL-8 protein secretion.

RESULTS: The attachment of *S. typhimurium* ATCC 14028 to INT407 intestinal epithelial cells was inhibited significantly by *B. lactis* HN019. *B. lactis* HN019 could be internalized into the INT-407 cells and attenuated IL-8 mRNA level at both baseline and *S. typhimurium*-induced pro-inflammatory responses. IL-8 secretion was reduced while IL-1β and TNF-α mRNA expression level remained unchanged at baseline after treated with *B. lactis* HN019.

CONCLUSION: *B. lactis* HN019 does not up-regulate the intestinal epithelium expressed pro-inflammatory cytokine, it showed the potential to protect enterocytes from an acute inflammatory response induced by enteropathogen.

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Key words: Probiotics; Intestinal epithelium; Enteropathogen; Immunomodulation; Adhesion

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INTRODUCTION

The intestinal tract acts as a reservoir for the intestinal microbiota that exert both harmful and beneficial effects on human health; intestinal microbiota contains an extraordinarily complex variety of metabolically active bacteria and fungi which interact with the host's epithelial cells and provide constant antigenic stimulation to the mucosal immune system[1]. The intestinal epithelium presents the

first line of defense against invading or attaching bacteria. In addition to serving as a physical barrier to microbial penetration, the intestinal mucosa is the main interface between the immune system and the luminal environment[2]. Intestinal epithelial cells (IECs) appear as an essential link in communicating with the immune cells in the underlying mucosa and the microflora in the lumen *via* the expression of many mediators. These mediators included antibacterial peptides such as defensins[3], mucins (MUC3), and chemokines and cytokines such as interleukin (IL)-8.

Under certain pathological circumstances, such as enteropathogens infection, the intestinal epithelium releases the chemokine IL-8 and other pro-inflammatory molecules that provoke an acute inflammatory response[4]. Therefore, a prolonged infection can result in a high level and protracted IL-8 release by IECs. The final outcome is a considerable infiltration of neutrophils that may perpetuate inflammation and eventually lead to cell damage, epithelial barrier dysfunction and pathophysiologic change of diarrhea[5].

For the protection against enteropathogen infections, the possibility of using food supplements containing probiotic bacteria has been recently considered. Probiotics are a group of live micro-organisms administered in adequate amounts which confer a beneficial effect on the health of the host[6]. Most probiotics found are resident members of the intestinal flora and are of major economic importance to the food industry. Several health-related effects associated with the intake of probiotics have been reported in different animal models as well as in human studies[7]. This bacterial community plays a pivotal role in human nutrition and health by promoting the supply of nutrients, preventing pathogen colonization and shaping and maintaining normal mucosal immunity[8]. While the precise mechanistic basis of the beneficial effects of probiotics is still obscure and will most likely vary depending on the strain and species used, a number of mechanisms have been suggested[9]. Protecting the host from enteropathogen colonization (barrier effects) and immunomodulatory effects toward host immune response have been demonstrated in humans and laboratory animals[10,11].

The interaction between probiotic strains and the intestinal epithelium is a key determinant for cytokine production by enterocytes, and probably the initiating event in probiotic immunomodulatory activity, as it occurs prior to the encounter with the immune system cells. It has been reported that several strains of probiotics belonging to *Bifidobacterium* and *Lactobacillus* are highly relevant to the prevention of the invasion of tissues by enteropathogens[12]. Moreover, by inhibiting the production of IL-8 in enterocytes, these strains are also found to be effective in modulating the pro-inflammatory response in IECs challenged by enteropathogens such as *Salmonella typhimurium* (*S. typhimurium*); such induction is species and strain specific[13,14].

Since the immunomodulatory properties are strain-specific[15-17], for each probiotic strain, profiles of the cytokines secreted by lymphocytes, enterocytes and/or DCs that come into contact with the strain should be established. Strains belonging to the *Bifidobacterium* are the most widely used probiotic bacteria, and are included in many functional foods and dietary supplements.

We focused our study mainly on how IEC respond to widely used probiotic bacteria. The aim of this work was to study the cytokine pattern induced by the interaction of probiotics with intestinal epithelium cells. Furthermore, whether probiotic bacteria still function after inactivation was evaluated. This study may contribute to verify the pro- and anti-inflammatory properties of the strain in question and define specific clinical applications.

MATERIALS AND METHODS

Bacterial strains and related preparations

A globally consumed commercial probiotic strain *Bifidobacterium lactis* (*B. lactis*) HN019 was studied in the experiment. The *B. lactis* HN019 powder was a gift from Danisco. Live strain was inoculated in De Man, ROGOSA and SHARPE (MRS) broth (Difco) supplemented with 0.05% (w/v) cysteine hydrochloride at 37℃ under anaerobic conditions for 24 h. The stationary-phase bacteria were harvested and Gram-stained to confirm purity. Heat-killed *B. lactis* HN019 were prepared by heating the bacteria at 80℃ for 20 min. Effectiveness of the heat-killing method was confirmed by plating the treated bacteria on MRS agar at 37℃ for 48 h. *S. typhimurium* ATCC 14028 was used as an enteropathogenic reference strain. *S. typhimurium* was cultured in Tryptone Soy broth (TSB) at 37℃ with shaking overnight.

Bacteria number was estimated by measuring the absorbance at 600 nm, and correlating the absorbance value to a standard curve of colony-forming units (CFU) on MRS agar (Merck) estimated in preliminary experiments.

For storage, liquid cultures of *B. lactis* HN019 grown for 24 h were frozen in 30% glycerol at -80℃; vacuum freeze-drying method was also applied.

Cell lines and media

INT-407 cells which derived from human intestinal epithelium, were purchased from American Type Culture Collection (ATCC strain CCL-6). Cells were grown in a humidified incubator at 37℃ under 5% CO_2. INT-407 cells were cultured in Dulbcco's Modifed Eagle Medium (DMEM) (GIBCO) containing 10% (v/v) heat-inactivated fetal bovine serum (Invitrogen), 50 U/mL penicillin, and 50 μg/mL streptomycin. The cells were routinely propagated in 25 cm^2 tissue culture flasks until they approached 80%-90% confluences. Subsequently, the cells were subcultured and the medium was replaced every two to three days in the culturing process.

Adhesion assays

Each adhesion assay was conducted in triplicate over three to five successive passages of INT-407 cells. Briefly, INT-407 cells were seeded on sterile six-well plates and grown to 80% confluence. The monolayers of INT-407 cells were rinsed three times with modified Hank's Balanced Salt Solution (mHBSS) without calcium and magnesium. Late exponential cultures of *B. lactis* HN019 or/

and *S. typhimurium* ATCC 14028 were adjusted at an optical density at 600 nm corresponding to 1×10^8 cells/mL. After rinsing, the bacterial cells were resuspended in DMEM and used for the adhesion assay. After incubation for 2 h and 6 h at 37℃ in the atmosphere of 5% CO_2 and 95% air, unattached bacteria were removed by washing the monolayers four times with sterile PBS. After detachment of the INT-407 cells from the plastic surface by incubation with 200 μL trypsin/EDTA per well (10 min, 37℃), the INT-407 cells and the adhesive bacteria were transferred into a 1.5 mL reaction tube. The wells were rinsed with 200 μL sterile PBS which was also transferred into the 1.5 mL-reaction tube.

Bacterial adhesion to INT-407 cells was evaluated by quantification of INT-407-bound bacteria with genus-specific primers *via* real-time polymerase chain reaction (PCR) as reported by Candela *et al*[18]. To quantify the bacterial cells by real-time PCR, the cell suspensions obtained from the adhesion assays were thawed at room temperature and, after mixing, an aliquot of 20 μL was transferred into a 0.2 mL-reaction tube and incubated for 10 min at room temperature with 3.8 μL of Trypsin inhibitor solution. Then the bacterial cells (Bifidobacterium and Salmonella) were specifically quantified by real-time PCR performed with the genus-specific primers listed in Table 1. pMD19 T-vector which carried genus-specific genes was constructed as internal standards.

Real-time PCR was performed in ABI 7500 instrument and SYBR Green I fluorophore was used to correlate the amount of PCR product with the fluorescent signal. Amplification was carried out in a 20 μL final volume containing 2 μL of cell suspension, 10 μL SYBR Premix Ex Taq (TAKARA), 0.2 μmol/L of each primer and 0.4 μL ROX Reference Dye Ⅱ. The experimental protocol consisted of the following programs: starting preincubation at 95℃ for 10s; amplification including 40 cycles of 95℃ for 5 s, and 60℃ for 34 s. Since the copy numbers of standard plasmids could be quantified, we amplified serial dilutions of the plasmids that represent bacteria number ranging from 1×10^7 to 1×10^3 CFU/μL.

The data reported represent mean values obtained in 3-5 independent experiments. Each experiment was performed in duplicate.

Preparation of samples for electron microscopy

INT-407 cells treated with *B. lactis* HN019 for 2 h and 6 h as previously described were washed three times with PBS buffer solution. Formaldehyde (40%) and glutaraldehyde (10%) were added for fixation. Ultrastructure of the cells was examined under transmission electron microscopy. The micrographs were produced at 5800 × and 13500 × magnification. Cells from the control group which had not been treated with bacteria were processed in the same way. Two samples from each group were analyzed.

INT-407 cells treatment

Monolayer of INT-407 cells were washed in PBS and supplemented with DMEM plus 2% FBS without antibiotics overnight before infection. *B. lactis* HN019 and *S. typhimurium* ATCC 14028 was harvested in stationary-phase, centrifuged and resuspended in fresh DMEM medium. After heat-killing treatment, viable *B. lactis* HN019, *S. typhimurium* ATCC 14028 and inactivated *B. lactis* HN019 were added into six-well plates for co-culture with INT-407 cells at a bacterial to epithelial cell ratio of 10:1. Untreated cells with normal medium were used as negative control. The plates were kept in humidified atmosphere containing 5% CO_2 for 2 h.

Table 1 Primer sequences of genus-specific genes used in adhesion assays

Primer name	Primer sequences (5′-3′)	Specificity	Ref.
BIF 164	GGGTGGTAATGCCGGATG	*Bifidobacterium*	[37]
BIF 662	CCACCGTTACACCGGGAA		
MINf	ACGGTAACAGGAAGCAG	*Salmonella*	[38]
MINr	TATTAACCACAACACCT		

Bacterial interference assays

The ability of *B. lactis* to inhibit binding of *S. typhimurium* to INT-407 cells was also assessed. INT-407 cells (1×10^6) seeded in six-well plates in antibiotic-free medium were incubated for 24 h. After replenishing the medium, the following assays were performed. (1) Coincubation of *B. lactis* HN019 and *S. typhimurium* ATCC 14028 with INT-407 cells for 2 h; (2) INT-407 cells were pretreated with 10:1 *B. lactis* HN019 for 2 h prior to infection with 10:1 *S. typhimurium* ATCC 14028 for further 2 h; (3) INT-407 cells were pre-stimulated with 10:1 *S. typhimurium* ATCC 14028 for 2 h prior to retreatment with 10:1 *B. lactis* HN019 for further 2 h; and (4) Untreated INT-407 cells were used as baseline control.

RNA extraction

Following bacterial treatment, cell culture supernatants were removed and washed three times with sterile D-Hank's. INT-407 cells collected for RNA preparation were immediately lysed in TRIZOL Reagent (Invitrogen). Total RNA was isolated from the lysed cells according to the manufacturer's instructions. Purification of RNA was performed by RNase-free DNase and standard phenol chloroform extraction method. The quantity of RNA was evaluated by spectrophotometric analysis and the quality of RNA samples was determined based on the A260:A280 ratio. All RNA samples were further analyzed by agarose gel electrophoresis to check for the integrity of 5S, 18S and 28S rRNA.

Real-time RT-PCR analysis

Total RNA (2 μg) was reverse-transcribed to yield cDNA using SuperScript Ⅲ First-Strand Synthesis System (Invitrogen) as the manufacture's protocol for the following experiments. Primers targeting four genes of interest were designed for detecting mRNA expression. Real-time RT-PCR was performed in an ABI 7500 system (Applied Biosystems) using SYBR Premix Ex Taq as mentioned

Table 2 Primer sequences used for quantitative real-time PCR analysis

Gene	Primer sequences (5′-3′)	Product size (bp)
IL-1β	ACTCACTTAAAGCCCGCCTG TCAGAATGTGGGAGCGAATG	80
IL-8	CAAGAGCCAGGAAGAAACCAC TGCAGAAATCAGGAAGGCTG	101
TNF-α	TCTTCTCGAACCCCGAGTGA CCTCTGATGGCACCACCAG	150
β-actin	TGTTACAGGAAGTCCCTTGCC AATGCTATCACCTCCCCTGTG	101

PCR: Polymerase chain reaction; IL: Interleukin; TNF: Tumor necrosis factor.

above. Calculations were performed using β-actin as an internal standard. Primer sequences are shown in Table 2. All reactions were performed in triplicate. Relative gene expression data was determined by the $2^{-\Delta\Delta CT}$ method as previously described[19]. Fold changes > 2 or < -0.5 was defined as significantly changed between treated and untreated cells.

IL-8 ELISA

Subconfluent or confluent INT-407 cells were incubated with varying groups of bacteria: (1) Cells were incubated with *B. lactis* HN019 or heat-killed *S. typhimurium* ATCC 14028 at a bacterial to epithelial cell ratio of 10:1 for 12 h; (2) Confluent monolayers of INT-407 cells were stimulated with 10 ng/mL LPS (derived from *S. typhimurium,* Sigma) for 12 h; (3) INT-407 cells were co-incubated with *B. lactis* HN019 (multiplicity of infection = 10) and LPS (10 ng/mL) simultaneously for 12 h; (4) Pretreated with 10:1 *B. lactis* HN019 for 2 h prior to stimulation with 10 ng/mL LPS for further 12 h; (5) Pre-stimulated with 10 ng/mL LPS for 2 h, and then treated with 10:1 *B. lactis* HN019 for further 12 h; and (6) Untreated cells were used as negative control.

Cells from all treatments were washed three times with PBS. Immunoreactive IL-8 protein levels in cell-culture supernatants were quantified using an ELISA Duo-Set kit (R & D Systems) according to the manufacturer's protocol.

Statistical analysis

Values were given as mean ± SD of triplicate measurements. Statistical analyses were performed using unpaired two-tailed Student's *t* test. *P* value < 0.05 was considered to be statistically significant.

RESULTS

Inhibitory effects of B. lactis HN019 on S. typhimurium ATCC 14028 attachment

The *B. lactis* HN019 strains in this study were evaluated with regard to their ability to inhibit the attachment of pathogenic *S. typhimurium* ATCC 14028 to INT-407 cells. Adherence activity of *B. lactis* HN019 and/or *S. typhimurium* ATCC 14028 was evaluated by a real-time PCR as shown in Figure 1.

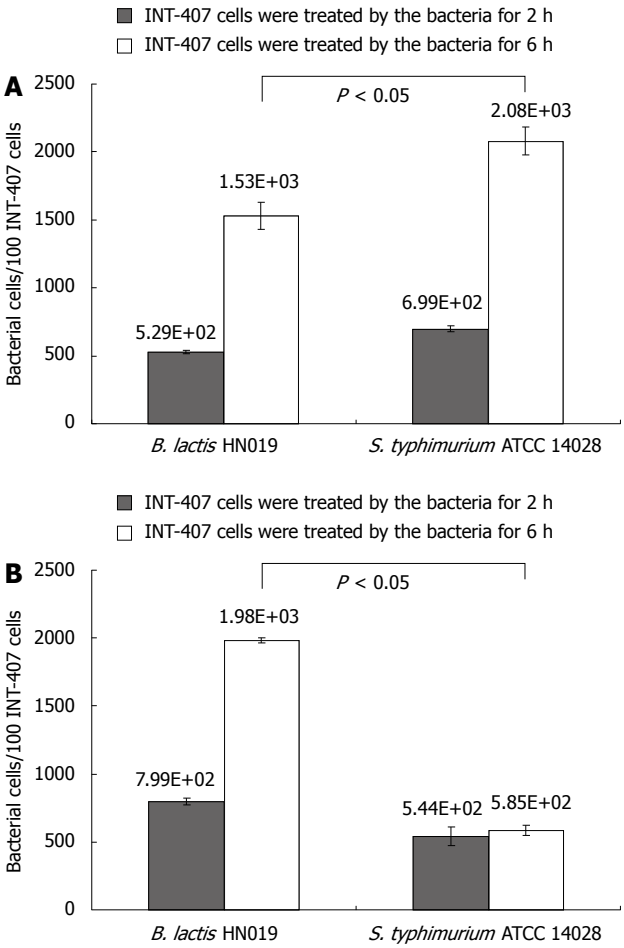


Figure 1 Adherence of *Bifidobacterium lactis* (*B. lactis*) HN019 and *Samonella typhimurium* (*S. typhimurium*) ATCC 14028 to INT-407 cells. A: Number of bacteria bound to 100 INT-407 cells obtained in assays contain each single strain; B: Number of bacteria bound to 100 INT-407 cells obtained in assays contain *S. typhimurium* ATCC 14028 plus *B. lactis* HN019. The error bars represent ± SD of the mean values.

Candela *et al*[18] defined bacterial strains as non-adhesive with less than 5 bacterial cells adhered to one cell. Following this adhesion score, both *B. lactis* HN019 and *S. typhimurium* ATCC 14028 could effectively adhere to INT-407 cells. Moreover, the adhesive capacity of *B. lactis* HN019 was not higher than *S. typhimurium* ATCC 14028 when treated with either strain alone, showing the adhesion values of 5.29×10^2 and 6.99×10^2 bacterial cells/100 INT-407 cells after 2 h while 1.53×10^3 and 2.08×10^3 bacterial cells/100 INT-407 cells after 6 h.

Nevertheless, the adhesion values obtained in co-treated assays showed that the *B. lactis* HN019 could significantly decrease the attached number of *S. typhimurium* ATCC 14028 from 2.08×10^3 to 5.85×10^2 bacterial cells/100 INT-407 cells after 6 h treatment, and the number of attached *B. lactis* HN019 was not affected by the existence of *S. typhimurium* ATCC 14028.

Transmission electron microscopy studies

In these studies we observed that *B. lactis* HN019 was able to interact with the epithelial cells of small intestine (Figure 2), and it was interesting that the *B. lactis* HN019

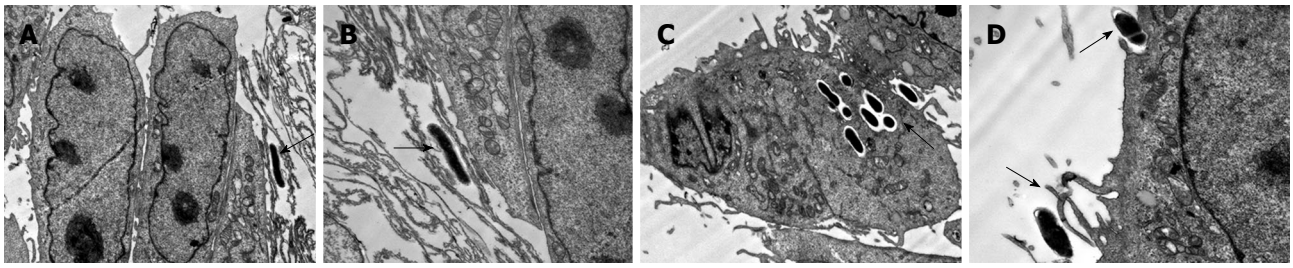


Figure 2 Transmission electron micrograph of INT-407 cells after stimulation with *B. lactis* HN019 (arrows). A: Stimulation for 2 h (× 5800); B: Stimulation for 2 h (× 13500); C: Stimulation for 6 h (× 5800); D: Stimulation for 6 h (× 13500).

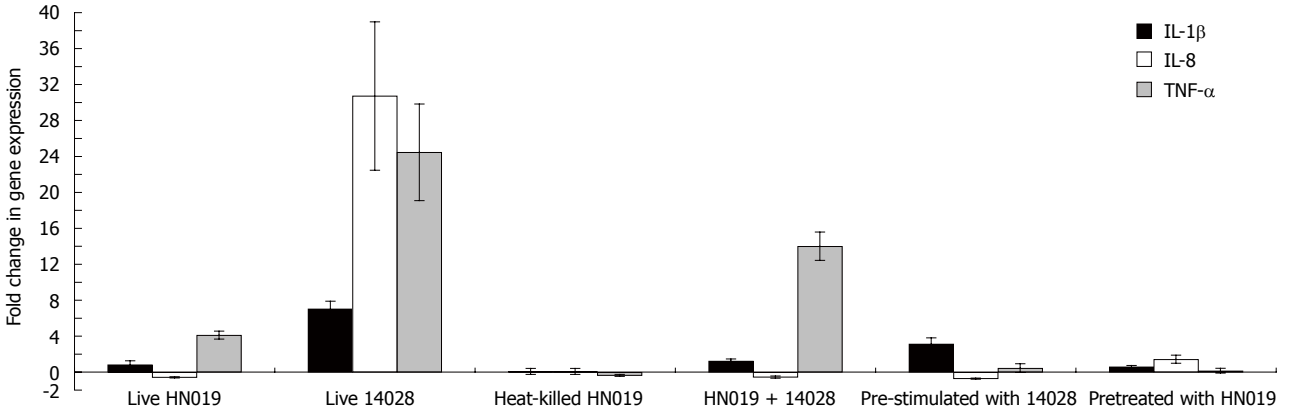


Figure 3 Effects of different bacterial treatment on expression of three genes assessed by real-time polymerase chain reaction. IL: Interleukin; TNF: Tumor necrosis factor.

could be internalized into the INT-407 cells after 6 h treatment.

Inflammatory gene expression following bacteria incubation

Inflammatory gene expression in bacteria-treated INT-407 cells was analyzed with real-time PCR. The results showed that live *B. lactis* HN019 had no effect on gene expression of IL-1β, but could up-regulate TNF-α expression and down-regulate IL-8 expression. Heat-killed *B. lactis* HN019 had no effect on gene expression of IL-1β, IL-8 and TNF-α. Pathogenic *S. typhimurium* ATCC 14028 could significantly up-regulate the mRNA level of pro-inflammatory cytokines. Although co-incubation with *B. lactis* HN019 and *S. typhimurium* ATCC 14028 could induce the gene expression of TNF-α and IL-1β, the extent is significantly lower than in those stimulated with *S. typhimurium* ATCC 14028 alone (Figure 3).

In bacterial interference assays, IL-1β, IL-8 and TNF-α mRNA expression was not changed at baseline after pre-treated with *B. lactis* HN019, and addition with *S. typhimurium* ATCC 14028 also did not activate these pro-inflammatory cytokines expression. For groups pre-stimulated with *S. typhimurium* ATCC 14028, *B. lactis* HN019 lowered the *S. typhimurium* ATCC 14028 induced IL-1β, IL-8 and TNF-α levels from 8.73, 47.97 and 9.3 folds to 2.6, 0.89 and 0.37 folds as compared with the untreated group. The results are summarized in Figure 3.

Through either incubating the INT-407 cells with *B. lactis* HN019 and *S. typhimurium* ATCC 14028 simultaneously or pre-treating with each strain respectively, the pro-inflammatory cytokines induced by *S. typhimurium* ATCC 14028 could be suppressed by *B. lactis* HN019. We therefore compared the inhibitory effects of these three groups and found that for IL-1β, the inhibition rate of *B. lactis* HN019 pretreated group was much lower than the other two groups. For IL-8, there was no significant difference among the three groups. For TNF-α, the inhibition rate of co-treated group was much lower than the other two groups (Figure 4).

IL-8 secretion by INT-407 cells following bacteria incubation

To assess the effects of probiotic strain on IL-8 secretion, INT-407 cells were incubated with live or heat-killed *B. lactis* and *S. typhimurium* and/or lipopolysaccharide. The experiments were performed 2-5 times in replicate; the results are demonstrated in Figure 5.

Firstly, we tested the effects of live *B. lactis* HN019, heat-killed *B. lactis* HN019 and *S. typhimurium* ATCC 14028, LPS derived from *S. typhimurium* on IL-8 secretion. The results showed that both live and dead *B. lactis* HN019 decreased IL-8 levels (844 ± 64 pg/mL and 897 ± 80 pg/mL) compared with the untreated group (1963 ± 77 pg/mL). Co-culturing with heat-killed *S. typhimurium* ATCC 14028 induced no change in IL-8 concentration. However, stimulation of INT-407 cells with LPS resulted in a significant increase of IL-8 production (3104 ± 80 pg/mL).

Secondly, in order to assess the anti-inflammatory

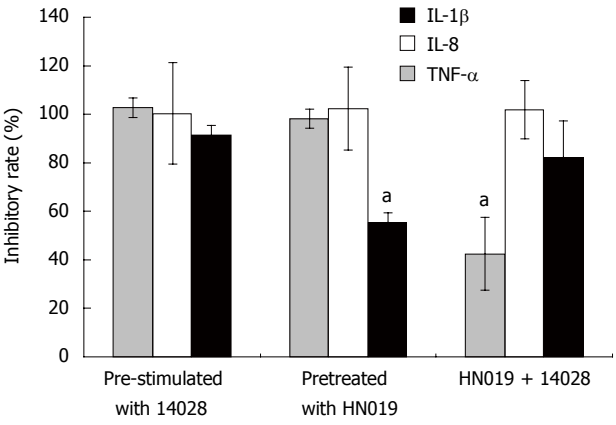


Figure 4 Inhibitory effects of *B. lactis* HN019 on gene expression of pro-inflammatory cytokines. $^{a}P < 0.05$ *vs* other treatment methods.

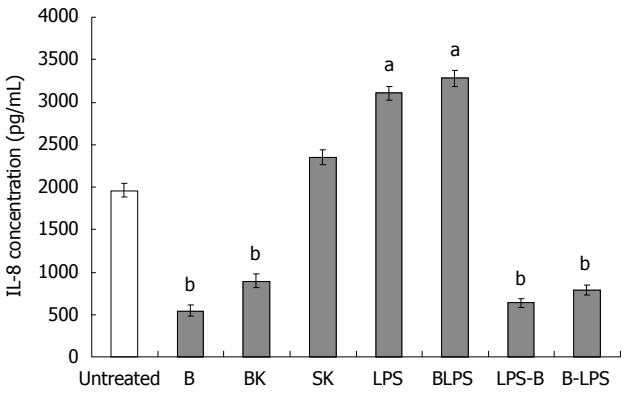


Figure 5 Bacteria modulate IL-8 secretion at baseline. B: *Bifidobacterium lactis* HN019; BK: Heat-killed *B. lactis* HN019; SK: Heat-killed *Salmonella typhimurium* ATCC 14028; LPS: Lipopolysaccharide (10 ng/mL); BLPS: Co-incubation with *B. lactis* HN019 and LPS; LPS-B: Treated with *B. lactis* HN019 after pretreatment with LPS for 2 h; B-LPS: Treated with LPS after pretreatment with *B. lactis* HN019 for 2 h. The data are expressed as pg/mL and represent the mean ± SE. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* untreated monolayers.

properties of the probiotic strain, the IL-8 production was evaluated by incubating INT-407 cell monolayers with *B. lactis* HN019 in the presence of LPS. As presented in Figure 5, IL-8 protein level secreted by the cells noticeably increased after co-incubated with LPS and probiotic strain compared with untreated group. However, the probiotic strain could decrease the LPS-induced IL-8 expression, and the cells pretreated with probiotic strain could also remain at a low level IL-8 secretion even after re-stimulated with LPS as compared with untreated group. This suggests that this probiotic strain *B. lactis* HN019 exerted anti-inflammatory effects on the epithelium by down-regulating the secretion of IL-8.

DISCUSSION

The role of probiotic bacteria derived from normal intestinal microbiota in the physiology of the gastrointestinal tract is not completely understood. In particular, the mechanisms underlying the benefits from biotherapy with probiotics have not been sufficiently elucidated. *B. lactis* HN019 is a world-wide consumed probiotic strain which is associated with several health-related immunomodulatory effects: such as reducing the severity of pathogen infection and enhancing immunity in the elderly[20-22]. Nevertheless, the mechanism is yet to be known. This is the first study on the differential pro-inflammatory cytokine responses of intestinal epithelium under conditions employing live and heat-killed *B. lactis* HN019. Furthermore, the enteropathogen exclusion effect of *B. lactis* HN019 was not evaluated.

In the present study, we used a rapid, accurate and sensitive method for studying bacteria adhesion. Provided specific primers for bifidobacteria are known, our method allows the quantification of this organism's cell number adhering to INT-407 cells which is used as a model in our investigation. A further important advantage of the real-time PCR based method is its efficacy in detecting and quantifying different bacterial genera and species simultaneously adhering to an epithelial cell monolayer. This method can be useful for probiotic strain selection. By this method, we found that *B. lactis* HN019 exerted a strong adhesive activity to human intestinal epithelial cells INT-407. The ability to adhere to the intestinal epithelium represents a significant prerequisite for the transient intestinal colonization of probiotic bacteria[23]. Bifidobacteria are normal gastrointestinal flora of many animals and have been shown to be adherent to a variety of epithelial cells *in vitro*[18,24,25]. However, adherence to INT-407 cells is seldom reported. This adherence to INT-407 cells may be mediated *via* fibronectin binding[26]. It is known that probiotic strains have the ability to counteract the pathogenic bacteria invasion[27,28]. In particular, the bifidobacteria strain tested is effective in inhibiting the adhesion of pathogenic *S. typhimurium* ATCC 14028 to INT-407 cells. This behavior is a fundamental prerequisite for probiotic bacteria to exert antagonistic activities against enteropathogens[29]. Such inhibitory effects of bifidobacteria can be explained by the mechanisms of competition for common adhesive sites or producing antibacterial lipophilic factor(s)[30]. In addition, it is reported that *bifidobacteria* could produce a proteinaceous factor that inhibits *in vitro* adherence of an enterotoxigenic *E. coli* strain to gangliotetraosylceramide molecules, which are physiological constituents of the mammalian intestinal epithelial surface[31].

Interactions of *B. lactis* HN019 and INT-407 cells observed under transmission electron microscope (TEM) indicated that the bacteria are able to contact with the epithelial cells of the small intestine. Moreover, the *B. lactis* HN019 could be internalized into INT-407 cells. It has been described that pathogens (bacteria, virus and parasites) can cross the mucosal barrier through different routes: transcellular route, paracellular route across cells for the tight junctions and *via* M cells[32]. The *B. lactis* used in this study was originally isolated from a human host. Therefore, it may be that this strain can internalize into epithelial cells because of its host specificity. We considered that such internalization may be of potential risks if the safety status of the internalized bacteria were not carefully assessed, especially the translocation capacity which correlate with its ability to induce infections[33].

The colonization of the intestinal tract by enteropathogenic bacteria induces the activation of the inflammatory cascade, resulting in the epithelial cell secretion of IL-8 and other pro-inflammatory molecules and in the consequent recruitment of neutrophils and other inflammatory cells. IL-8 amplifies an ongoing acute immune response and provides a set of signals for the activation of mucosal inflammatory responses in the earliest phases of microbial invasion. In some cases, a massive and prolonged infiltration of neutrophils may perpetuate inflammation and ultimately lead to cell damage, epithelial barrier dysfunction and pathophysiologic change of diarrhea. In the present study, INT-407 cells behaved like human enterocytes and could secrete IL-8 either constitutively or after stimulation with physiological agonists. Probiotic strains presented differences in their capacity to augment IL-8 expression, however, some strains seemed to rather decrease epithelial-cell production of IL-8[15-17,34-36]. Our results showed a robust IL-8 expression of INT-407 cells induced when exposed to *S. typhimurium*. However, live and heat-killed *B. lactis* HN019 not only functionally modulate the epithelium by inhibiting the constitutive mRNA level of IL-8 and attenuating *S. typhimurium*-induced *IL-8* gene expression, but also protect the INT-407 cells from IL-8 protein production activated by LPS. The same results were discovered on *TNF-α* and *IL-1β* gene expression except for up-regulation of TNF-α expression by live *B. lactis* HN019, but the *S. typhimurium* could induce much higher TNF-α expression than live *B. lactis* HN019. High concentrations of IL-1β and TNF-α cause cachexia, tissue injury, disseminated intravascular coagulation and shock. Therefore, the control of secretion of these pro-inflammatory cytokines *in vivo* is very important. *B. lactis* HN019 has a beneficial effect in converting the infection-induced excessive cytokine expression and helping maintain an immunological balance. These data indicated that INT-407 displayed immunological quiescence when exposed to both live and dead *B. lactis* HN019. *B. lactis* HN019 can modulate epithelial responses to limit inflammatory signals and markedly inhibit the inflammatory response induced by pathogen.

We used three approaches to detect the influence of probiotic strain on the pathogenic strain, and found that although the *B. lactis* HN019 could reduce the expression of pro-inflammatory cytokines, the inhibitory effect was different depending on the treatment methods. Pre-stimulated INT-407 cells with *S. typhimurium* ATCC 14028 could strongly induce a high-level of pro-inflammatory cytokine expression and such up-regulation could be reversed to a normal level after re-treatment with probiotic *B. lactis* HN019. This treatment achieved the maximum inhibitory rate. These results indicated that *B. lactis* HN019 may be more effective as adjunctive therapeutic agent than as preventive agent in inflammatory diseases.

In conclusion, our studies revealed that as a probiotic strain, *B. lactis* HN019 could modulate immune system towards anti-inflammatory action and exclude enteropathogenic adhesion, thus contributing to the homeostasis of the intestinal epithelium. This finding will offer, in the near future, new therapeutic means to counteract the inflammatory disorders observed in human inflammatory bowel disease.

ACKNOWLEDGMENTS

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COMMENTS

Background

Bifidobacterium lactis (*B. lactis*) HN019 is a world-wide consumed probiotic strain and several health-related effects of the strain have been reported, especially the immunomodulatory effect. The immunomodulatory properties are strain-specific, and the interaction between probiotics and the intestinal epithelium is the initiating event in immunomodulatary activity. The authors focused their studies on how intestinal epithelial cells (IEC) respond to widely used probiotic bacteria to explore the mechanism of the immunomodulatory effects of *B. lactis* HN019.

Research frontiers

Studies in probiotics are one of the hottest research fields at present. The interactions between bacteria and the host may reveal the mechanisms of bacteria on the host. This study may contribute to better applications of probiotic bacteria.

Innovations and breakthroughs

This is the first study on different pro-inflammatory cytokine responses of intestinal epithelium under conditions employing *B. lactis* HN019. The authors used a rapid, accurate and sensitive method for studying bacteria adhesion and found that *B. lactis* HN019 exerted a strong adhesive activity to human intestinal epithelium cells and the bifidobacteria strain tested was effective in inhibiting the adhesion of pathogen. The authors also indicated that *B. lactis* HN019 could modulate epithelial responses to limit inflammatory signals and markedly inhibit the inflammatory response induced by pathogen.

Applications

The study revealed that as a world-wide consumed probiotic strain, *B. lactis* HN019 could modulate immune system towards anti-inflammatory action and exclude enteropathogenic adhesion. These findings may help with better applications of the *B. lactis* HN019 and will contribute to offering new therapeutic means to counteract the inflammatory disorders observed in human inflammatory disease.

Peer review

The manuscript by Liu *et al* describes the adherence and immuno-modulatory properties of *B. lactis* HN019 against INT-407 intestinal epithelial cells, and additionally reports the anti-*S. typhimurium* properties of this probiotic strain. This is an interesting manuscript which has been well executed.

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BRIEF ARTICLE

Establishment of a human hepatoma multidrug resistant cell line *in vitro*

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Abstract

AIM: To establish a multidrug-resistant hepatoma cell line (SK-Hep-1), and to investigate its biological characteristics.

METHODS: A highly invasive SK-Hep-1 cell line of human hepatocellular carcinoma, also known as malignant hepatoma was incubated with a high concentration of cisplatin (CDDP) to establish a CDDP-resistant cell subline (SK-Hep-1/CDDP). The 50% inhibitory dose (IC_{50}) values and the resistance indexes [(IC_{50} SK-Hep-1/CDDP)/(IC_{50} SK-Hep-1)] for other chemotherapeutic agents and the growth curve of cells were all evaluated using cell counting kit-8 assays. The distribution of the cell cycles were detected by flow cytometry. Expression of acquired multidrug resistance P-glycoprotein (MDR1, ABCB1) and multidrug resistance-associated protein 1 (MRP1, ABCC1) was compared with that in parent cells by Western blotting and immunofluorescence combined with laser scanning confocal microscopy.

RESULTS: The SK-Hep-1/CDDP cells (IC_{50} = 70.61 ± 1.06 μg/mL) was 13.76 times more resistant to CDDP than the SK-Hep-1 cells (IC_{50} = 5.13 ± 0.09 μg/mL), and CDDP-resistant cells also demonstrated cross-resistance to many anti-tumor agents such as doxorubicin, 5-fluorouracil and vincristine. Similar morphologies were determined in both SK-Hep-1 and SK-Hep-1/CDDP groups. The cell cycle distribution of the SK-Hep-1/CDDP cell line exhibited a significantly increased percentage of cells in S (42.2% ± 2.65% *vs* 27.91% ± 2.16%, $P < 0.01$) and G2/M (20.67% ± 5.69% *vs* 12.14% ± 3.36%, $P < 0.01$) phases in comparison with SK-Hep-1 cells, while the percentage of cells in the G0/G1 phase decreased (37.5% ± 5.05% *vs* 59.83% ± 3.28%, $P < 0.01$). The levels of MDR1 and MRP1 were overexpressed in the SK-Hep-1/CDDP cells exhibiting the MDR phenotype.

CONCLUSION: Multiple drug resistance of multiple drugs in the human hepatoma cell line SK-Hep-1/CDDP was closely related to the overexpression of MDR1 and MRP1.

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Key words: Hepatoma; Cell line; Multidrug resistance; *In vitro*; Cisplatin

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INTRODUCTION

Multidrug resistance (MDR)[1] is a major obstacle in the chemotherapy of cancer patients and is also one of the reasons for tumor relapse and metastasis. Hepatocellular

carcinoma (HCC) is one of the most common gastrointestinal tumors worldwide, accounting for 70%-90% of primary liver cancers[2], with a high degree of malignancy and poor prognosis. A majority of patients are surgically unresectable at the time of diagnosis, and even for those where surgery is possible, the risk of recurrence is extremely high. Consequently, chemotherapy is an important treatment for most HCC patients. However, HCC responds poorly to chemotherapy owing to acquired MDR[3,4]. The ability to overcome this MDR is a major concern in clinical oncology in successfully treating HCC[5].

Cisplatin (CDDP) is a key drug that is widely used for cancer chemotherapy, but the effectiveness of CDDP for HCC is unsatisfactory because of MDR. To elucidate the CDDP-resistant mechanism in HCC *in vitro*, we established a CDDP-resistant SK-Hep-1 cell line and analyzed its biological behavior.

MATERIALS AND METHODS

Drugs and chemicals

CDDP, doxorubicin (DOX), penicillin, streptomycin, propidium iodide (PI) and RNase A were obtained from the Sigma-Aldrich Chemical Co (St.Louis, MO, USA). Vincristine (VCR) was obtained from Shenzhen Main Luck Pharmaceuticals Inc. (Shenzhen, China). 5-fluorouracil (5-FU) was obtained from Shanghai Xudong Haipu Pharmaceutical Co. Ltd (Shanghai, China). Dulbecco's modified Eagle's medium/high glucose (DMEM/H), fetal bovine serum (FBS) and Trypsin were purchased from Invitrogen (Carlsbad, CA, USA). The phospho-glycoprotein (P-gp, MDR1) mouse monoclonal antibody, multidrug resistance-associated protein 1 (MRP1)-specific mouse anti-human antibody and FITC-conjugated goat anti-mouse IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). MitoTracker Red CMXRos dye purchased from Invitrogen (Molecular Probes, Invitrogen Corp.). Cell Counting Kit-8 (CCK-8) was obtained from Dojindo Molecular Technologies (Dojindo, Japan). The cell culture supplies were purchased from Corning Life Sciences (Lowell, MA, USA). Anti-tumor agents were prepared extemporaneously in complete culture medium immediately prior to use *in vitro*.

Cells and cell culture

The human hepatoma cell line, SK-Hep-1, was obtained from the Cell Culture Center, Institute of Basic Medical Sciences within the Chinese Academy of Medical Sciences. SK-Hep-1 cells were cultured in DMEM/H containing 10% (v/v) FBS, penicillin (200 U/mL), streptomycin (100 μg/mL), and were incubated at 37℃ in a humidified incubator with an atmosphere of 50 mL/L CO_2.

Establishment of a CDDP-resistant SK-Hep-1 subline *in vitro*

SK-Hep-1/CDDP was produced by exposing SK-Hep-1 cells to CDDP repeatedly at a single high concentration over a period of 24 h. Briefly, SK-Hep-1/CDDP was selected by a procedure consisting of 6 pulse drug treatments with 5 μg/mL CDDP, with SK-Hep-1 cells (1×10^6) plated onto a 25 cm^2 culture flask in each pulse treatment. When cells were growing exponentially, they were exposed to CDDP for 24 h. The majority of the cells were dead following 24 h exposure to CDDP. The treated cells were then washed with 0.01 mol/L phosphate-buffered saline (PBS) and cultured in CDDP-free growth medium. After 1-2 d, the dead cells were washed out with PBS and fresh medium again added. The resistant subclones were isolated by limiting dilution. After 4 weeks' incubation at 37℃ in a humidified incubator containing 50 mL/L CO_2, the cells recovered at an exponential rate and were then subcultured at a density of 1×10^6 cells/25 cm^2 flasks. Once cells reached 60%-70% confluence, the cells were preserved for further study as described above. The CDDP-resistant subclone was established 6 mo after the treatment was initiated, then the resistant phenotype developed. For maintenance of CDDP-resistant cells, the SK-Hep-1/CDDP cells were grown in the presence of 0.01 μg/mL CDDP. Before experimentation, SK-Hep-1/CDDP cells were maintained in a CDDP-free culture medium and subcultured at least 3 times. The MDR characteristics of these SK-Hep-1/CDDP cells were tested using various concentrations of anticancer drugs including CDDP, DOX, VCR and 5-FU.

CCK-8 cell proliferation and cytotoxicity assay

Cell proliferation assays were performed with CCK-8 according to the manufacturer's instructions. Cells (2×10^3) were seeded into each well of a 96-well plate and cultured in 100 μL of DMEM/H supplemented with 10% FBS. At the indicated time points, medium was exchanged for 110 μL of DMEM/H with CCK-8 reagent (10 μL CCK-8 and 100 μL DMEM/H), and the cells were incubated for 2 h. Absorbance was measured for each well at a wavelength of 450 nm, with the reference wavelength set at 600 nm. An increase or decrease in absorbance values at 450 nm in the experimental wells relative to the initial value indicated cell growth or death, respectively. Cell growth was monitored every 24 h over 5 d, and was repeated in 6 wells. All assays were carried out independently in triplicate.

The effects of chemotherapeutic agents on the growth of SK-Hep-1 and SK-Hep-1/CDDP cells were also evaluated with CCK-8. Cells (2×10^3/well) were seeded into 96-well plates in 100 μL of DMEM/H with 10% FBS incubated at 37℃ in a humidified atmosphere containing 50 mL/L CO_2. After 24 h the medium was aspirated, and exchanged with media containing a test chemotherapeutic agent at various concentrations. After incubation for 24 h at 37℃, the drug-containing growth medium was replaced with 110 μL medium containing CCK-8 reagent. After 2 h, the absorbance was read at 450 nm with a reference wavelength at 600 nm. Six wells were used for each drug concentration and the experiment was replicated 3 times. The IC_{50} was calculated by SPSS13.0 (SPSS Inc., Chicago, IL, USA). The lower the IC_{50} value, the higher the potency against cell proliferation.

Cell cycle analysis

Control and CDDP-resistant cells were harvested, washed twice with ice-cold PBS (pH 7.2) and fixed in ethanol: PBS (9:1) at -20℃ for at least 30 min. The fixed cells were then washed twice with ice-cold PBS and stained with 50 mg/mL PI in the presence of 25 mg/mL RNase A. Cell cycle phase distribution was analyzed in 3 different experiments using a COULTER® EPICS™ XL™ flow cytometer (Beckman Coulter Inc., Brea, CA, USA). Data from 50 000 events/sample were collected and analyzed using CellQuest software (BD Biosciences, San Jose, CA, USA).

Immunolabeling and quantification

For immunofluorescence, cells were grown on glass slides in 6-well culture plates. Two days later, MitoTracker Red in DMEM/H (200 nmol/L) was added to live cells for 30 min at 37℃, and washed twice with ice-cold PBS (0.01 mol/L, pH 7.2). The cells were fixed for 30 min in 4% ice-cold paraformaldehyde followed by permeabilization with 0.3 mg/mL Triton X-100 for 30 min at room temperature. Fixed, permeabilized cells were washed twice with 0.01 mol/L PBS and incubated with blocking buffer containing goat serum for 30 min at room temperature. Cells were washed twice with 0.01 mol/L PBS again and the expression of MDR1 was determined using the mouse monoclonal anti-P-gp antibody (1:100), Expression of MRP1 was determined using the mouse monoclonal anti-MRP1 antibody (1:100). Negative controls for immunofluorescence involved replacing the primary antibodies with an IgG isotype control. The primary antibody incubations were carried out in blocking buffer overnight at 4℃. After incubation with primary antibodies, cells were washed 3 times with 0.01 mol/L PBS, and incubated with secondary FITC-conjugated goat anti-mouse antibody (1:50) in the dark for 1.5 h at 37℃. Cell nuclei were counterstained with DAPI (4,6-diamidino-2-phenylindole; Sigma-Aldrich, St. Louis, MO, USA) solution (1 μg/mL in PBS) for 5 min at room temperature. After washing with 0.01 mol/L PBS, cells were mounted on glass slides using Antifade Mounting Medium (Beyotime Institute of Biotechnology, Jiangsu, China). Confocal laser scanning microscopic analysis of immunolabeling was performed on a Leica TCS SP2 confocal laser imaging system (Leica Microsystems, Wetzlar, Germany). To ascertain localization of the mitochondria, cells were stained with MitoTracker Red CMXRos dye. A merged double image of green MDR1 or MRP1 and MitoTracker Red was obtained. To allow quantitative comparisons of the relative fluorescence intensity of cells between groups, cells were chosen on a random basis and scanned at more than 3 points for analysis. Software Image-Pro Plus Version 6.0 (MediaCybernetics, Bethesda, MD, USA) was used to count the mean value of the fluorescence intensity. Average green fluorescence intensity per cell was determined as the quantity of P-gp or MRP1 protein expression.

Detection of P-gp and MRP1 protein by Western blotting

Total protein was collected from cultured SK-Hep-1 and SK-Hep-1/CDDP cells and the concentration was measured using a BCA Protein Assay Kit (Beyotime Institute of Biotechnology, Jiangsu, China). The protein was denatured in lithium dodecyl sulfate (LDS) sample buffer (106 mmol/L Tris-HCl, 141 mmol/L Tris base pH 8.5, 0.51 mmol/L EDTA, 10% glycerol, 2% LDS, 0.22 mmol/L SERVA blue G250, 0.175 mmol/L phenol red, 0.1 mmol/L 2-mercaptoethanol) for 5 min at 95℃, electrophoresed on a 7.5% SDS-PAGE and blotted onto 0.2 μm PVDF membranes (Roche, Indianapolis, IN, USA). Membranes were blocked with 5% (w/v) dry milk in TBS-T (Tris-buffered saline containing 0.05% Tween 20) for 1 h at room temperature and incubated overnight at 4℃ with antibodies against P-gp (1:500) or MRP1 (1:100). After incubation with the respective primary antibodies, the membranes were washed 3 times for 5 min in TBS containing 0.05% Tween-20, and then the membranes were exposed to species-specific horseradish peroxidase-labeled secondary antibodies (ZhongShan Goldenbridge Biotechnology, Beijing, China) at 37℃ for 1 h, and developed using the ECL plus Western blotting reagent with visualization on X-ray films. The expression of β-actin was detected as an internal control. The band intensity on X-ray films was quantified using Gel-Pro Analyzer software. The ratio of the band intensity for P-gp/β-actin and MRP1/β-actin was used as the relative expression level for P-gp and MRP1 protein, respectively.

Statistical analysis

All experiments were run in triplicate, and the results are given as mean ± SD. Statistical analyses were performed using either an analysis of variance (ANOVA) or Student *t* test. The difference was considered statistically significant when the *P* value was less than 0.05. All statistical analyses were carried out with SPSS 13.0 software.

RESULTS

Establishment of a cisplatin-resistant SK-Hep-1/CDDP cell line

We established CDDP-resistant SK-Hep-1 (SK-Hep-1/CDDP) cells by pulse exposure of SK-Hep-1 cells to high concentrations of CDDP for short periods over 24 h. This caused a marked change in cellular morphology with many elongated cell dendrites and deposits of intracytoplasmic vacuoles observed, and cells membrane became less clear, or cells died. These effects were clearly observed after 24 h treatment with CDDP at 5 μg/mL. The surviving cells recovered exponentially and then were further selected by a procedure consisting of 6 pulse drug treatments with 5 μg/mL CDDP. The SK-Hep-1-resistant subclone was established 6 mo after the treatment was initiated. Microscopic observation revealed that the SK-Hep-1/CDDP cells (Figure 1B) adopted a spindle shape, similar to that of the parent cells (Figure 1A). The sensitivity of SK-Hep-1 and SK-Hep-1/CDDP cells to various concentrations of CDDP was determined by CCK-8 assay. As shown in Table 1, IC_{50} values for CDDP on SK-Hep-1 and SK-Hep-1/CDDP cells were 5.13 ±

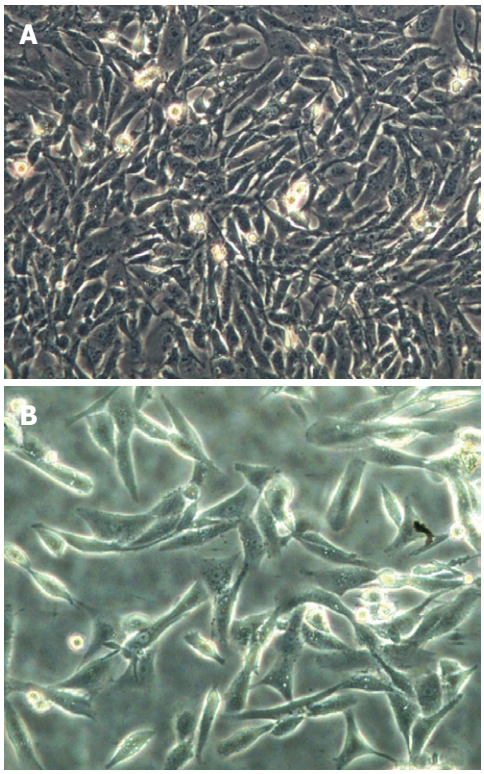


Figure 1 The cell shape of SK-Hep-1 (A) cell line and SK-Hep-1/CDDP (B) cell line (× 100). SK-Hep-1: A highly invasive human hepatoma cell line with a epithelial-like morphology; SK-Hep-1/CDDP: A cisplatin-resistant cell line from the parental cell line.

Table 1 IC_{50} and RI values of SK-Hep-1 and SK-Hep-1/CDDP cells

Drug (μg/mL)	IC_{50} (mean ± SD)		RI
	SK-Hep-1	SK-Hep-1/CDDP	
CDDP	5.13 ± 0.09	70.61 ± 1.06[b]	13.76
DOX	0.74 ± 0.04	4.13 ± 0.23[b]	5.58
VCR	0.76 ± 0.02	2.28 ± 1.06[b]	3.12
5-FU	12.49 ± 0.27	52.79 ± 3.85[b]	4.23

[b]Significant difference in comparison with the parental cells ($P < 0.01$). SK-Hep-1 and SK-Hep-1/CDDP cells were exposed to indicated concentrations of CDDP, DOX, VCR and 5-FU for 24 h and determined using the CCK-8 assay. The IC_{50} values were calculated. Values of 3 independent experiments are represented as mean ± SD. Resistance index (RI) = (IC_{50} SK-Hep-1/CDDP cells)/(IC_{50} SK-Hep-1 cells). CDDP: Cisplatin; IC_{50}: 50% inhibitory dose; DOX: Doxorubicin; VCR: Vincristine; 5-FU: 5-fluorouracil.

0.09 μg/mL and 70.61 ± 1.06 μg/mL, respectively. SK-Hep-1/CDDP cells were 13.76-fold more resistant to CDDP than the parent cells. We also compared the cross-resistance to other anticancer drugs (DOX, VCR, 5-FU) between the parent and CDDP-resistant cells, with our results indicating that the SK-Hep-1/CDDP cells also had cross-resistance to DOX, VCR and 5-FU.

Cell growth and cell cycle

The growth curves for SK-Hep-1 cells and the CDDP-resistant SK-Hep-1 subline are shown in Figure 2A, and the cell cycle distribution of each cell line is shown in Figure 2B and C, and Table 2. The resistant cells grew

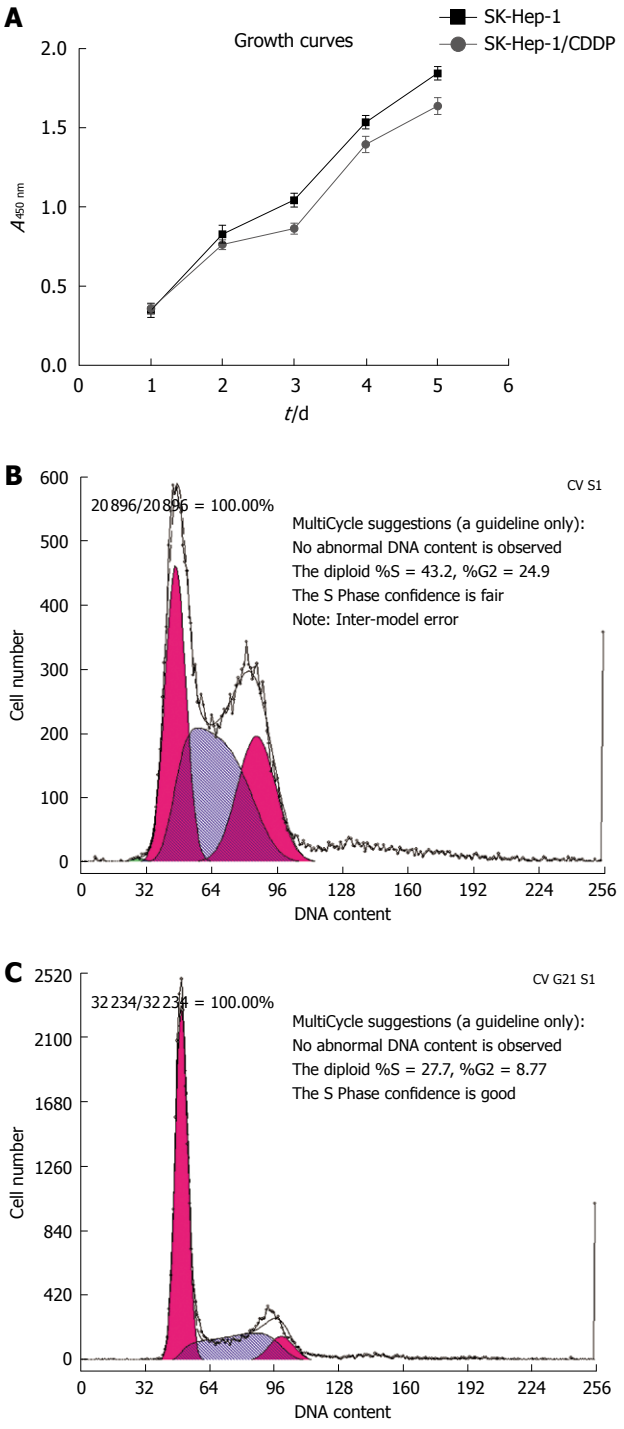


Figure 2 Growth curves of 2 cell lines, SK-Hep-1 and SK-Hep-1/CDDP (A), flow cytometric analysis of cell cycle distribution of SK-Hep-1/CDDP (B) and SK-Hep-1 (C).

more slowly than the the parent cells ($P < 0.05$). Cell cycle analysis revealed that the number of SK-Hep-1/CDDP cells in the G0/G1 phase decreased, accompanied by an increased proportion of cells in the S phase and G2/M phases ($P < 0.05$, Table 2).

Evaluation of MDR1 and MRP1 by laser scanning confocal microscope

We examined the expression of the ATP-binding cassette (ABC) transporters[6], MDR1 and MRP1, by immunofluo-

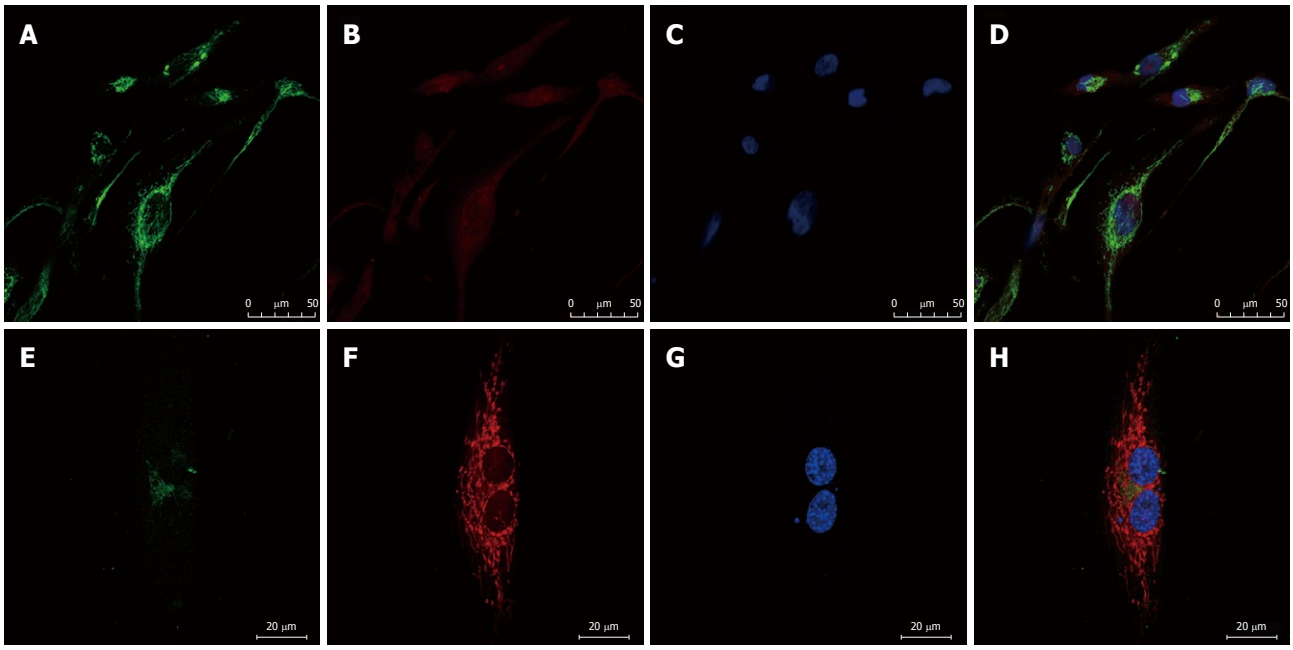


Figure 3 Laser scanning confocal microscopy for MDR1/P-gp in resistance SK-Hep-1/CDDP (A-D) and parent SK-Hep-1 (E-H) cell lines. SK-Hep-1/CDDP (A) and SK-Hep-1 (E) cells were simultaneously stained for the anti-P-gp antibody-FITC (green), (B) and (F) were stained with mitochondria (red), (C) and (G) immunofluorescence images for the nuclei (blue), (D) and (H) the expression of the merged image. MDR1: Multidrug resistant protein 1; P-gp: Phospho-glycoprotein.

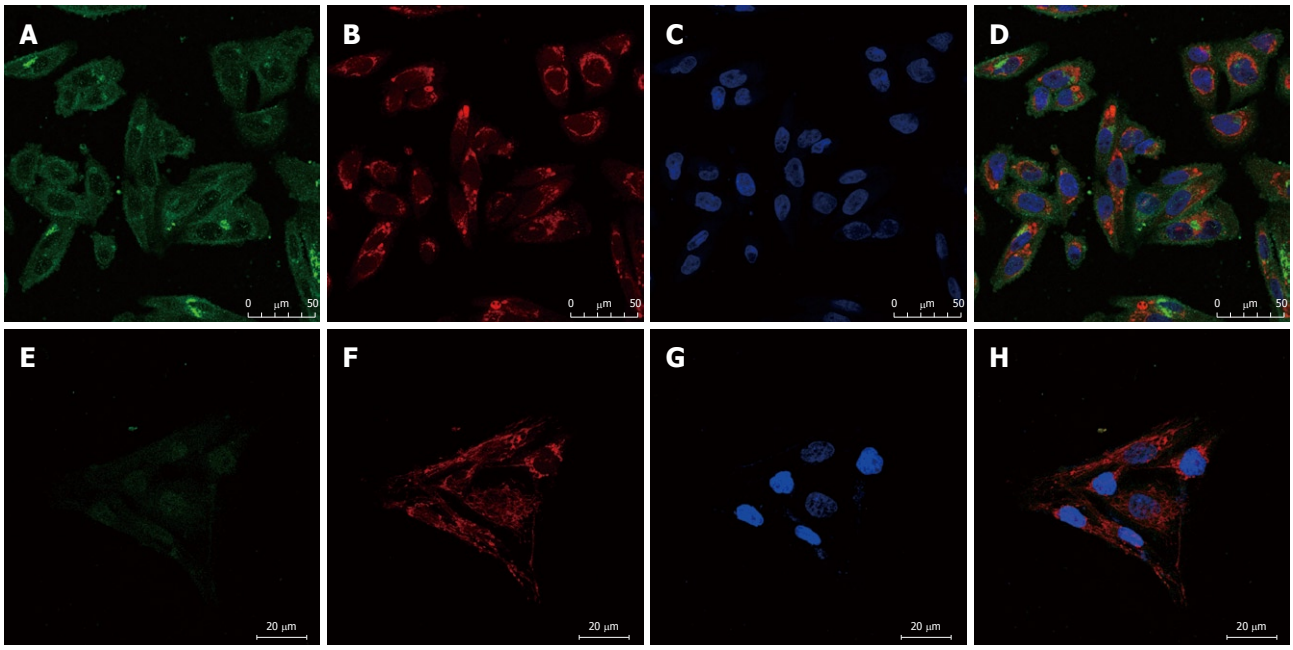


Figure 4 Overexpression of MRP1 in resistant SK-Hep-1/CDDP (A-D) and SK-Hep-1 (E-H) cell lines. Immunofluorescence images for MRP1 (A and E, green), mitochondria (B and F, red), and nuclei (C and G) on the same section were merged to illustrate areas of overlap (D and H). MRP1: Multidrug resistance-associated protein 1.

rescence. Confocal microscopic analysis confirmed that expression of both MDR1 and MRP1 was elevated in SK-Hep-1/CDDP and SK-Hep-1 cells (Figures 3 and 4), MDR1 or MRP1 appeared to localize around the nuclear membranes. To further assess the localization of MDR1 and MRP1, we performed a double stain with MitoTracker Red and DAPI. The green fluorescein fluorescence image was ubiquitous (MDR1 or MRP1). Figure 3 shows the relative level of MDR1/P-gp (green fluorescence) in SK-Hep-1/CDDP (Figure 3A-D) and parent SK-Hep-1 cell (Figure 3E-H) as viewed by confocal microscopy. Figure 4 shows the relative level of MRP1 (green fluorescence) in SK-Hep-1/CDDP (Figure 4A-D) and parent SK-Hep-1 cell (Figure 4E-H).

The results of the quantitative assessment are shown in Table 3. CDDP-resistant cells demonstrate pronounced high levels of green fluorescence and high mean gray density when compared with SK-Hep-1 cells ($P < 0.05$).

Table 2 Cell cycle distribution of SK-Hep-1 and SK-Hep-1/CDDP cells (%)

Cell	G0/G1	S	G2/M
SK-Hep-1	59.83 ± 3.28	27.91 ± 2.16	12.14 ± 3.36
SK-Hep-1/CDDP	37.50 ± 5.05[a]	42.20 ± 2.65[a]	20.67 ± 5.69[a]

[a]$P < 0.05$, resistant cells *vs* SK-Hep-1. SK-Hep-1/CDDP in G0/G1 phase decreased accompanied by an increased proportion of cells in the S phase and G2/M phase.

Table 3 Semi-quantification of P-gp and MRP1 immunofluorescence (mean ± SD)

Cell	P-gp (n = 3)	MRP1 (n = 3)
SK-Hep-1	20.57 ± 6.59	3.75 ± 0.02
SK-Hep-1/CDDP	64.79 ± 12.4[a]	35.78 ± 6.17[a]

[a]$P < 0.05$, resistant cells *vs* SK-Hep-1.

P-gp and MRP1 expression in cells by Western blotting

The expression of P-gp/MDR1 and MRP1 in SK-Hep-1/CDDP cells was evaluated by Western blotting analyses. These proteins were detected in the parental SK-Hep-1 and SK-Hep-1/CDDP cells. The levels of P-gp and, MRP1 were significantly higher in the SK-Hep-1/CDDP resistant cells than in the SK-Hep-1 cells ($P < 0.01$, Figure 5). We considered it important to confirm the maintenance of MDR1 and MRP1 expression and its functionality for our culture conditions. The gray intensity of P-gp/β-actin was 0.58 ± 0.02 for SK-Hep-1 and 1.32 ± 0.04 for SK-Hep-1/CDDP ($P < 0.01$, $n = 3$). The gray intensities of MRP1/β-actin were 0.24 ± 0.01 and 0.59 ± 0.03 for SK-Hep-1 and SK-Hep-1/CDDP cells, respectively ($P < 0.01$, $n = 3$).

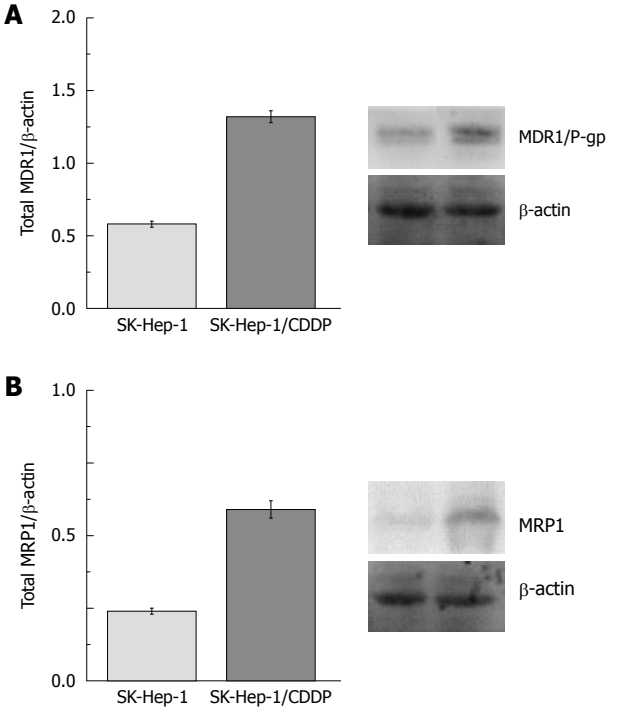


Figure 5 Representative Western blotting of total protein extracts from SK-Hep-1 and SK-Hep-1/CDDP cells. A: Left: The relative level of MDR1/β-actin in each cell line in the bar graph ($P < 0.05$, $n = 3$). Right: Western blotting analysis of MDR1/P-gp; B: Left: The relative level of MRP1/β-actin in each cell line in the bar graph ($P < 0.05$, $n = 3$). Right: Expression level of total MRP1 protein in SK-Hep-1 and SK-Hep-1/CDDP cells. The data are representative of those obtained in 3 other experiments.

DISCUSSION

Intrinsic and acquired resistance to multiple chemotherapeutic drugs is a major obstacle in the clinical treatment of cancer, and the mechanisms responsible for MDR remain unclear. Tumor-derived cell lines in which MDR is induced by the intermittent exposure to drugs[7,8] are frequently used models to study these mechanisms. In order to elucidate the molecular mechanisms of multiple chemotherapeutic drugs resistance in HCC, we established a CDDP-resistant hepatoma cell line as model for investigating chemotherapy resistance.

In this study, the CDDP-resistant subline SK-Hep-1/CDDP was induced by high-concentration, short-duration drug treatment[9]. SK-Hep-1/CDDP cells exhibited strong resistance to CDDP, 13.76 times greater than in SK-Hep-1 cells. The SK-Hep-1/CDDP cells showed significant changes in cell growth compared with SK-Hep-1, growing at a slower rate and exhibiting changes in the cell cycle following the development of drug resistance. The proportion of SK-Hep-1/CDDP cells in the G2/M and S phases increased significantly. Meanwhile SK-Hep-1/CDDP cells also demonstrated cross-resistance to multiple, structurally diverse chemotherapeutic agents, such as DOX, VCR and 5-FU. These resistant phenotypes were stable and the IC_{50} values and resistance index demonstrated no significant change over a 3-mo period in drug-free medium.

HCC resistance to chemotherapeutic treatment is possibly related to the overexpression of MDR proteins belonging to the ABC family. Recent studies showed that HCC expressed *MRP1*, *MDR1*, *MRP3* and breast cancer resistance protein (BCRP/ABCG2), and these might confer tumor cells with a MDR phenotype[10,11]. Some reports stated that MRPs were more likely to be candidates to mediate chemo-resistance in HCC than MDR1[12,13]. *MDR1*, *MRP1* and *MRP2* genes are well known as ABC transporters associated with CDDP-resistance, as these proteins cause the efflux of many anticancer agents that have different mechanisms. Our study revealed that both a CDDP-resistant subline and its parental cells expressed MDR1, but the subline cells overexpressed MDR1 and MRP1.

In summary, although chemotherapy-resistant tumor cell lines have some disadvantages, they do provide model systems where all biological parameters can be controlled and assessed. We established a CDDP-resistant human hepatoma cell line, which provided the basis for further study of resistant mechanisms and reversal of clinical HCC drug resistance.

COMMENTS

Background

Drug resistance, in particular multidrug-resistance (MDR), is still the major cause of anticancer chemotherapy failure. The most important factor of medicating a MDR phenotype is the increased activity of the membrane-embedded drug extrusion pump, MDR1/P-glycoprotein (MDR1/P-gp or ABCB1). The mechanism of the reverse MDR phenotype is thought to involve direct binding to P-gp and displacement of the anticancer drugs, which enhances intracellular toxic efficacy of these agents. Analysis of novel chemotherapy-resistant cell lines may duplicate as far as possible the treatment conditions used *in vivo*.

Research frontiers

Hepatocellular carcinoma (HCC) responds poorly to chemotherapy owing to MDR. Recent studies have shown the tumors derived from the colon, kidney, or adrenal cortex, and HCC exhibited overexpression of MDR1/P-gp. This overexpression results in a primary MDR phenotype of these cancers. Tumor-derived cell lines are one of the most important tools for investigation of the biological mechanisms directly leading to drug resistance in patients. Today, the experimental search for drug resistant mechanisms that are clinically relevant targets whose circumvention can improve cancer therapy is still ongoing.

Innovations and breakthroughs

The model SK-Hep-1/CDDP cell line can be used as an *in vivo* model to investigate the molecular mechanisms involved in MDR-related genes of hepatocarcinoma and to explore the targeted approaches for overcoming MDR in tumor cells.

Applications

The SK-Hep-1/CDDP cells can be employed as a model system as its biological parameters can be controlled and assessed, and novel biomarkers of chemotherapy resistance can be identified. The methods described in this study and the results presented might provide the basis for advanced studies in this field.

Peer review

The manuscript is interesting and could be useful. The establishment of a human hepatoma multidrug-resistance cell line *in vitro* presents interesting results. The data show that a cisplatin-resistant hepatoma cell line increased MDR1 comparing with control cells, and expressed MRP1. They concluded that this fact conferred cross-resistance to the cell line.

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CASE REPORT

Diagnosis of ruptured superior mesenteric artery aneurysm mimicking a pancreatic mass

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Abstract

Aneurysms and pseudoaneurysms of the superior mesenteric artery are potentially lethal and should be treated as urgently as possible. In a 52-year-old man with occasional epigastric pain, we accidentally discovered a superior mesenteric artery aneurysm that was ruptured with spontaneous tamponade in the uncinate process and in the head of the pancreas. The ruptured aneurysm had a heterogeneous appearance due to its thrombotic and hemorrhagic content, and it simulated a voluminous mass in the head and uncinate process of the pancreas, associated with mild dilatation of the main pancreatic duct. Recent advances in multidetector computed tomography and magnetic resonance imaging have enabled radiologists to develop a correct diagnosis of mesenteric aneurysms and pseudoaneurysms of the visceral branches of the abdominal aorta, and to differentiate this diagnosis from that of pancreatic or peripancreatic masses; angiography is currently used to confirm a diagnosis and to develop therapeutic treatments.

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Key words: Superior mesenteric artery; Magnetic resonance imaging; Computed tomography; Ruptured aneurysm

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INTRODUCTION

Aneurysms of the visceral branches of the abdominal aorta are rare and potentially life-threatening, with a documented prevalence of 0.1%-2%[1]. The superior mesenteric artery is the third most common site of splanchnic aneurysm[2]. Recent advances in computed tomography (CT) vascular imaging and magnetic resonance angiography have provided a highly accurate representation of abdominal splanchnic vessels[3]. Multi-detector computed tomography (MDCT) with 3D post-processing allows for an accurate representation of vascular course and caliber. Thanks to its high-contrast resolution, magnetic resonance imaging (MRI) is used to determine the composition of aneurysmatic masses.

The aim of this case report is to describe the imaging features of a ruptured superior mesenteric artery aneurysm, which created a giant hematoma and mimicked a pancreatic mass, with mild dilatation of main pancreatic duct.

CASE REPORT

A 52-year-old man with intermittent epigastric pain was admitted to our hospital. He had undergone cholecystectomy and left colonic resection for cancer of the large intestine 4 and 2 years earlier, respectively. Preoperative liver ultrasonography and chest X-rays performed during previous hospitalization were normal.

During his last hospital admission, the patient underwent laboratory tests that revealed a mild increase in serum pancreatic amylase and lipase activity (129 and 116 UI/L, respectively). Coagulation tests were normal, and clinical examination did not reveal significant results. The patient was scheduled for abdominal CT. MDCT showed an 8.2 cm × 6.6 cm mass in the area of the uncinate process of the pancreas (Figure 1A). A post-contrast infusion MDCT scan showed enlargement of the proximal portion of the superior mesenteric artery, which had a transverse diameter of 2.2 cm. Multiplanar reconstructions of MDCT images indicated communication of the mass with the enlarged superior mesenteric artery (Figure 1B). The artery was englobed into the mass, which resulted in its complete obstruction. Liver, spleen and kidneys were normal, as was the small bowel, as shown by CT. CT did not show any relapse or residual sign in the area where surgical anastomosis of the colon had been performed.

Enhanced and unenhanced MRI was performed to investigate the mass composition. T1-weighted MR images showed heterogeneous high signal content (Figure 1C); a low peripheral "rim sign" on T1 and T2 images was revealed. Post-gadolinium images confirmed the enlarged superior mesenteric artery and its communication with the mass; there was no significant contrast enhancement in the mass itself. The initial diagnostic hypothesis was of a giant mass of the pancreas, which involved the superior mesenteric artery. The radiologist who performed the examinations described "an expansive lesion in the pancreatic area involving the proximal tract of the superior mesenteric artery", whereas the mass was caused by development and fissuration of the aneurysm into the uncinate process of the pancreas.

Angiography confirmed dilatation of the superior mesenteric artery, without any sign of bleeding. The patient underwent surgical intervention with resection of the thrombotic clot-filled aneurysm (Figure 2), which ruptured with spontaneous tamponade in the pancreatic region and formed a giant hematoma; bowel vitality was good during the provocative occlusive test, and subsequent ligation of the superior mesenteric artery was performed. Distal pancreatectomy was also required for the retropancreatic extension of the hematoma. MDCT performed 6 mo later did not show any complications.

DISCUSSION

Early diagnosis and treatment of aneurysms and pseudoaneurysms of the superior mesenteric artery are essential to prevent them from rupturing and threatening patients' lives[4]. Etiology includes arteriosclerosis, fibromuscular dysplasia, connective tissue disease, infection, and arterial dissection[1,5]. Mycotic aneurysms affect the superior mesenteric artery in 33%-66% of cases[6]. Superior mesenteric artery pseudoaneurysms are often associated with chronic pancreatitis, or they can be a consequence of external or iatrogenic trauma[5,7].

We describe a case of superior mesenteric artery aneurysm which ruptured with spontaneous tamponade in the uncinate process and head of the pancreas, which mimicked a pancreatic mass on MDCT and MRI. The etiology of the superior mesenteric aneurysm in our patient has still not been established; a previous history of pancreatitis, vascular hypertension, connective disease or traumatic abdominal injury was not revealed.

It is known that, on unenhanced MDCT, a superior mesenteric aneurysm can simulate a pancreatic mass[8]; thrombotic or hemorrhagic material in the aneurysm sac provides heterogeneous signal intensity. In our case, the sac filled with blood clots showed a heterogeneous elevated signal on T1 images, which suggested the possibility of a mass with hemorrhagic content or a hematoma.

Without contrast enhancement during the arterial phase, it is very difficult to establish the vascular nature of aneurysms, which is why the radiologist made an incorrect diagnosis[9]. The radiologist who first described MDCT and MRI signs did not explore the vascular structures with multiplanar reconstructions and hypothesized aneurysm not as a primary diagnosis, but as a consequence of a giant mass in the pancreatic tissue. Initially, he did not consider the possibility of a fissurated aneurysm. Pre-angiographic evaluation of multiplanar reconstruction, performed with other colleagues, finally oriented him towards diagnosing a giant aneurysm ruptured into the uncinate process. Axial images and CT reconstructions showed the continuity of the mass and the superior mesenteric artery, and enabled radiologists to diagnose a vascular aneurysm.

There is a large group of peripancreatic structures that can simulate pancreatic cystic lesions, such as aneurysms of the superior mesenteric artery, superior mesenteric vein, hepatic artery and portal vein[10]. Large aneurysms compress the duodenum and biliary duct, thus causing obstructive jaundice[7]. In our case, magnetic resonance cholangiopancreatography (MRCP) showed the dislocation of the pancreatic tract of the main biliary duct, which was caused by the large ruptured aneurysmatic sac (Figure 1D). The choledochus had a caliber of approximately 1 cm, but this was not considered a significant feature since the patient had been cholecystectomized; a diameter of up to 1 cm is frequent in such patients. Dilatation of the main pancreatic duct has not been reported very frequently in the literature. MRCP showed moderate dilatation of the main pancreatic duct and mild ectasia of the side branches

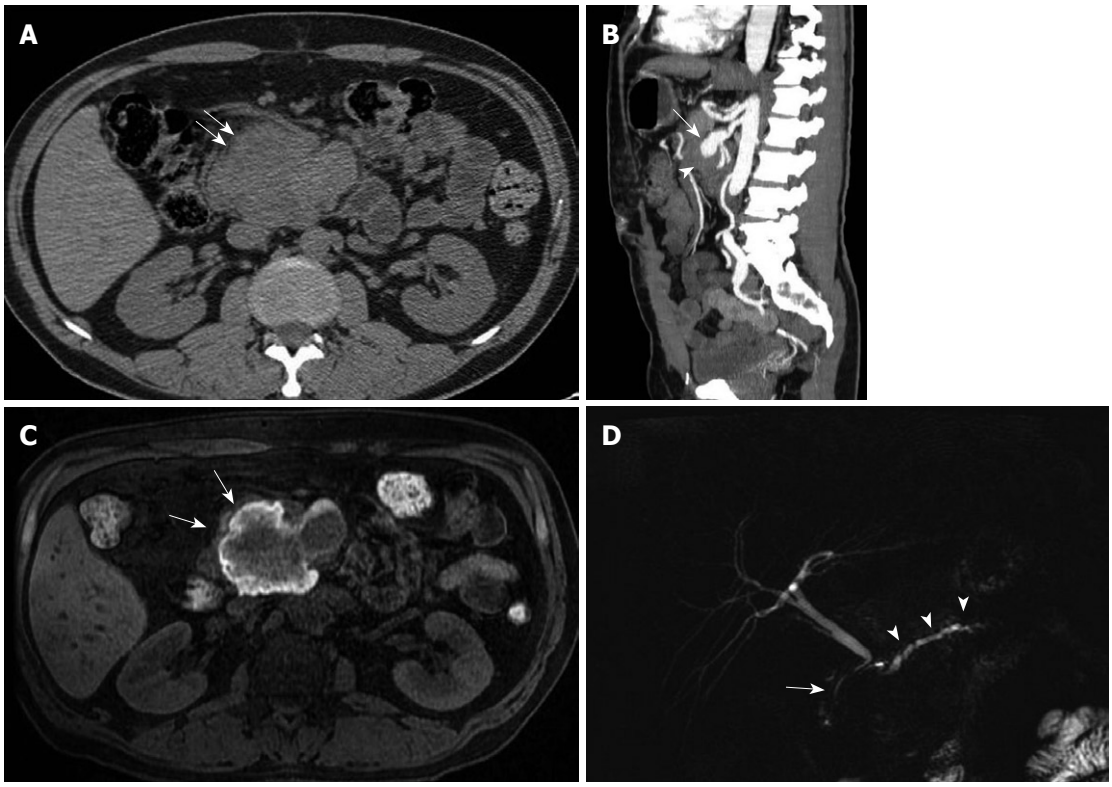


Figure 1 Images of the patient. A: Axial unenhanced multi-detector computed tomography (MDCT) shows a soft-tissue mass (arrows) in the area of the uncinate process of the pancreas. There is no sign of calcification or fluid level; B: Sagittal multiplanar reconstruction clearly shows the superior mesenteric artery (arrow) involved in a giant soft-tissue attenuated mass - the hematoma (arrowhead) - at the pancreatic level. After contrast administration, there was no significant enhancement in the hematoma, due to its thrombotic content; C: Magnetic resonance, axial unenhanced gradient echo T1-weighted imaging with fat suppression. The T1 hyperintense perilesional signal (arrows) was caused by the blood content; D: Magnetic resonance cholangiopancreatography (MRCP) shows dilatation of the main pancreatic duct (arrowheads); the choledochus (arrow) was compressed and dislocated, with mild narrowing of the lumen in the distal tract.

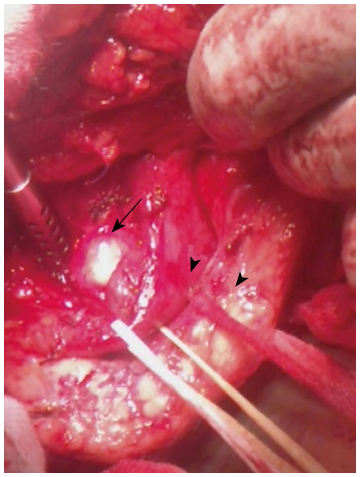


Figure 2 Surgical intervention. Intervention confirmed an aneurysm of the superior mesenteric artery, which ruptured with hematoma in the head of the pancreas. The figure shows the aneurysm (arrow) and superior mesenteric artery and vein (arrowheads).

of the main pancreatic duct (Figure 1D). The dilatation of the pancreatic duct was caused by the hemorrhagic sac, with evident dislocation of pancreatic parenchyma. Mild compression of the main pancreatic duct by the aneurysm sac can also explain minimally increased values of amylase and lipase activity discovered through laboratory tests, which initially physicians interpreted as symptoms of pancreatic disease.

Contrast administration is necessary to demonstrate a patent lumen, and suggests the correct diagnosis. Recent advances in CT technology have allowed for a more accurate multiplanar and 3D reconstruction, which shows the communication between the aneurysm sac and the blood vessels. High table-speed and rapid image acquisition during contrast injection clearly show the arterial anatomy. On CT, large aneurysms and pseudoaneurysms can simulate pancreatic pseudocysts with fluid content[11]. Blood clots and thrombotic deposits in aneurysms and pseudoaneurysms have a density similar to that of soft tissues or of high-density fluid collections. In some cases, pseudocysts have a hemorrhagic content, and on unenhanced CT scans, their density is the same as that of aneurysms.

MRI is very useful in terms of diagnosis because it provides radiologists with accurate information on mass composition: on T1-weighted images the presence of thrombotic and hemorrhagic material is easily demonstrated by the high-signal content of the mass. However, these features are not specific. Since hemorrhagic content on unenhanced T1 images can also be found in mucinous cystic neoplasms, a differential diagnosis is called for. Mucinous cystic lesions have variable signal intensity and sometimes proteinaceous material provides hyperintensity on T1-weighted images. Post-gadolinium images did not reveal internal septa and were very helpful in differentiating the diagnosis from that of cystic mucinous lesions.

In conclusion, aneurysms and pseudoaneurysms of the superior mesenteric artery at the pancreas level can be very insidious for radiologists because they can mim-

ick pancreatic masses. Significant advancements in 3D and multiplanar imaging software have made it possible to obtain high-resolution images of the abdominal aorta and its branches: radiologists need to keep in mind the diagnostic value of multiplanar reconstructions.

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CASE REPORT

Stroke and dilated cardiomyopathy associated with celiac disease

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Abstract

Celiac disease (CD) is manifested by a variety of clinical signs and symptoms that may begin either in childhood or adult life. Neurological symptoms without signs of malabsorption have been observed for a long time in CD. In this report, an 8-year-old girl with CD presented with rarely seen dilated cardiomyopathy and stroke. The girl was admitted with left side weakness. Her medical history indicated abdominal distention, chronic diarrhea, failure to thrive, and geophagia. On physical examination, short stature, pale skin and a grade 2 of 6 systolic murmur were detected. Muscle strength was 0/5 on the left side, and 5/5 on the right side. Coagulation examinations were normal. Tests for collagen tissue diseases were negative. Factor V Leiden and prothrombin GA20210 mutations were negative. Tandem mass spectrophotometry and blood carnitine profiles were normal. Brain magnetic resonance imaging and cerebral angiography showed an infarction area at the basal ganglia level. Examinations of serologic markers and intestinal biopsy revealed CD. We emphasize that in differential diagnosis of ischemic stroke, CD should be kept in mind.

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Key words: Celiac disease; Stroke; Cardiomyopathy; Children

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Doğan M, Peker E, Cagan E, Akbayram S, Acikgoz M, Caksen H, Uner A, Cesur Y. Stroke and dilated cardiomyopathy associated with celiac disease. *World J Gastroenterol* 2010; 16(18): 2302-2304 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i18/2302.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i18.2302

INTRODUCTION

Celiac disease (CD) is a disease of the small intestine caused by an immune response to ingested gluten. This response results in characteristic damage to the villi, leading to malabsorption[1]. CD is manifested by a variety of clinical signs and symptoms that may begin in either childhood or adult life. Neurological symptoms without malabsorption signs have been observed for a long time in CD. Epilepsy, bilateral occipital calcification, cerebellar ataxia, degenerative central nervous system disease, peripheric neuropathy, myopathy and, rarely, stroke were defined as neurologic manifestations[2]. Tissue transglu-

taminase enzyme is an auto-antigen, which is related to gluten-associated immune events[3]. In this case report, an 8-year-old girl with CD presented with rarely seen stroke.

CASE REPORT

An 8-year-old girl was admitted to our emergency department with weakness of the left lower and upper extremities. It was learnt that the patient had geophagia, abdominal distention and chronic diarrhea for 2 years. On physical examination, her body weight and height were 16 kg (-2 SD) and 110 cm (-2.6 SD), respectively. Blood pressure was 110/70 mmHg. She had pale skin and mucosa, and a grade 2 of 6 systolic murmur at the inferior left side of the sternum. Muscle strength was determined as 0/5 on the left upper and lower extremities and 5/5 on the right side. The laboratory analysis, hemogram, serum electrolytes, glucose, cholesterol, triglyceride levels, liver and renal function tests were within normal limits. Thyroid hormone values were also found to be within the normal range [free T_4: 1.4 ng/dL (normal range: 0.8-2.2 ng/dL), total T_4: 7.4 mg/mL (normal range: 5.5-12.8 mg/L), thyroid stimulating hormone: 1.2 mU/mL (normal range: 0.6-5.5 mU/mL), total T_3: 170 ng/dL (normal range: 119-218 ng/dL), free T_3: 3.2 pg/mL (normal range: 2.0-4.0 pg/mL)]. C-reactive protein was negative and erythrocyte sedimentation rate was 19 mm/h. Vitamin B12 and folate levels were normal. Prothrombin time and activated partial thromboplastin time were 13.6 s (normal range: 11-14 s) and 29 s (normal range: 31-40 s), respectively. Fibrinogen, protein C, protein S, factors V, Ⅶ, Ⅷ, Ⅺ, Ⅻ and antithrombin Ⅲ levels were within normal limits. Serologic tests for human immunodeficiency virus, lupus anticoagulants, antinuclear antibody, anti-double-stranded DNA, anticardiolipin antibody immunoglobulin (Ig) G and M serologies were negative. Factor V Leiden and prothrombin GA20210 mutations were not detected. The tandem mass metabolic disease screening panel and detailed blood carnitine profile were normal.

On brain magnetic resonance imaging, an infarction measuring 31 mm × 14 mm at the right basal ganglia level was seen (Figure 1). On cerebral angiography examination, a 1 cm segment occlusion was detected at the M2 branch of the right middle cerebral artery (Figure 2). Although dilated cardiomyopathy was identified on transesophagial and transthoracic echocardiographic examination, thrombus or vegetation was not seen. Serologic markers of CD were examined because of chronic diarrhea, abdominal distention, short stature, cerebral infarction and dilated cardiomyopathy, and anti-tissue transglutaminase IgA and IgG, and anti-endomysium IgA were found at a highly positive rate. Additionally, the examination of intestinal biopsy revealed CD. Duodenal biopsy showed villous atrophy with hyperplasia of the crypts and an increased intraepithelial lymphocyte count (above 40%).

A gluten-free diet and nadroparin calcium treatment were initiated and physiotherapy was performed. At the 18th day of hospitalization, the patient, whose symptoms had regressed, was discharged with a gluten-free diet, nadroparin calcium, co-enzyme Q and salicylate treatment. Symptoms resolved by the following 7th wk. Muscle strength at the left upper and lower extremities was 5/5, and other neurologic examinations were normal.

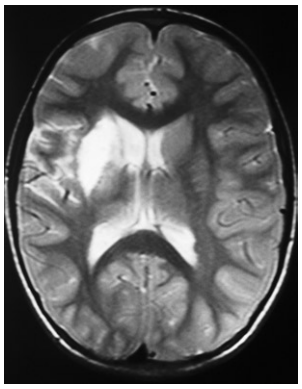


Figure 1 Brain magnetic resonance imaging shows an infarction area measuring 31 mm × 14 mm at the right basal ganglia level.



Figure 2 Cerebral angiography examination shows a 1 cm segment occlusion at the M2 branch of the right middle cerebral artery.

DISCUSSION

Classical findings of CD usually begin at 1-3 years of life. Toddlers and young children classically present with chronic diarrhea, vomiting, poor appetite, abdominal distension, abdominal pain, irritability, and failure to thrive some time after the introduction of gluten in the diet[1]. In adults, a variety of neuropsychiatric conditions, such as depression and anxiety, have been reported in individuals with CD[1]. In our case, abdominal distention, chronic diarrhea, geophagia, and short stature were observed. In addition, infarction and dilated cardiomyopathy were present.

The diagnosis of CD is established by positive results of serological testing and evidence of characteristic histopathology on intestinal biopsy[4]. If results of serologic testing are negative but clinical suspicion is high, intestinal biopsy should be performed. Characteristic histologic features of CD include varying degrees of villous atrophy, with hyperplasia of the crypts and an increased intraepithelial lymphocyte count[5]. Consistent with the literature, our case was positive for anti-tissue transglutaminase IgA and IgG, and anti-endomysium IgA, and duodenal biopsy examination showed villous atrophy

with hyperplasia of the crypts and increased intraepithelial lymphocyte count (above 40%).

Development of autoimmune diseases is one of the complications of the CD. Tissue transglutaminase enzyme is an auto-antigen that is related to gluten-associated immune events. Evidence of a central nervous system vasculitis was reported by Ozge *et al*[6] in a patient with recurrent stroke and CD. Pratesi *et al*[7] found that sera from patients with active CD contain IgA antibodies that reacted with human brain vessel structures, giving intense fluorescence. These antibodies were not present in sera from celiac patients on a gluten-free diet or non-celiac controls. They emphasized that this finding might be involved in the abnormal nervous system manifestations frequently described in association with CD. Tissue transglutaminase is the major auto-antigen in CD and is thought to maintain vascular endothelial integrity. Anti-endomysial IgA antibodies, demonstrated to be the same autoantibody as anti-transglutaminase, react with the cerebral vasculature, suggesting an autoimmune mechanism for CD-associated vasculopathy. Because CD is a potentially treatable cause of cerebral vasculopathy, serology (specifically for anti-tissue transglutaminase antibodies) should be included in the evaluation of cryptogenic stroke in childhood, even in the absence of typical gut symptoms.

Dilated cardiomyopathy is the most commonly seen type of cardiomyopathy. Regarding its etiology, genetic causes, endocrine disorders, collagen vascular diseases, drugs, congenital metabolism diseases, muscular dystrophies, structural heart diseases, acute and chronic myocarditis and toxins can be present. However, 50% of cases remain idiopathic. An increased incidence of CD in patients with idiopathic dilated cardiomyopathy as well as in patients with secondary cardiomyopathy has been reported recently[8]. In our case, dilated cardiomyopathy was diagnosed with echocardiography. However, dilated cardiomyopathy was not thought to be the primary factor causing the stroke, because there was no thrombus or vegetation at echocardiography, and regression of symptoms occurred with a gluten-free diet. In addition, in patients with a cryptogenic stroke, the presence of a patent foramen ovale should be evaluated. Transthoracic 2-dimensional echocardiography can generally show the atrial septum and the flap of the foramen ovale in infants and small children. Color Doppler flow across the atrial septum proves the presence of the foramen ovale. In older children and adults, transthoracic echocardiography does not visualize the atrial septum as well. Transesophageal echocardiography is preferred in patients where the atrial septum is inadequately visualized by transthoracic echocardiography. Older children and adults fall into this category. In addition to a patent foramen ovale, redundancy of the septum primum can also be seen. When the redundancy of the septum moves more than 1 cm, it is called an atrial septal aneurysm. In the presence of a patent foramen ovale in patients who have had a prior stroke, an atrial septal aneurysm confers an increased risk for a subsequent neurologic event[9,10]. In our patient, a patent foremen ovale was not found in both transthoracic and transesophageal echocardiographic examination. Therefore, a patent foremen ovale was not thought to be a cause of the stroke in our patient.

In conclusion, the cause of ischemic stroke in our case is thought to be multifactorial. We suggest that CD was a primary factor in its etiology, secondary to a contribution from dilated cardiomyopathy. In conclusion, we emphasize that in the differential diagnosis of ischemic stroke, CD should be kept in mind.

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CASE REPORT

Primary endoscopic approximation suture under cap-assisted endoscopy of an ERCP-induced duodenal perforation

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Abstract

Duodenal perforation during endoscopic retrograde cholangiopancreatography (ERCP) is a rare complication, but it has a relatively high mortality risk. Early diagnosis and prompt management are key factors for the successful treatment of ERCP-related perforation. The management of perforation can initially be conservative in cases resulting from sphincterotomy or guide wire trauma. However, the current standard treatment for duodenal free wall perforation is surgical repair. Recently, several case reports of endoscopic closure techniques using endoclips, endoloops, or fully covered metal stents have been described. We describe four cases of iatrogenic duodenal bulb or lateral wall perforation caused by the scope tip that occurred during ERCP in tertiary referral centers. All the cases were simply managed by endoclips under transparent cap-assisted endoscopy. Based on the available evidence and our experience, endoscopic closure was a safe and feasible method even for duodenoscope-induced perforations. Our results suggest that endoscopists may be more willing to use this treatment.

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Key words: Duodenal perforation; Endoscopic retrograde cholangiopancreatography; Endoscopic therapy; Endoclip

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Lee TH, Bang BW, Jeong JI, Kim HG, Jeong S, Park SM, Lee DH, Park SH, Kim SJ. Primary endoscopic approximation suture under cap-assisted endoscopy of an ERCP-induced duodenal perforation. *World J Gastroenterol* 2010; 16(18): 2305-2310 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i18/2305.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i18.2305

INTRODUCTION

Although MR cholangiopancreatography has almost completely replaced endoscopic retrograde cholangiopancreatography (ERCP) in the diagnosis of pancreato-biliary diseases, the risk of ERCP complications has increased as therapeutic endoscopists have taken on increasingly more complex cases, particularly at tertiary referral centers[1]. The frequency of duodenal perforation is 0.5%-2% of patients[2]. However, because of a relatively high mortality rate of 16%-18%, all duodenal perforations should be treated immediately after diagnosis[2-5].

Traditionally, the standard treatment for traumatic or iatrogenic duodenal perforation is surgical closure. Recently, endoscopic trials of perforation management have increased and successful primary repair of duodenal perforation using the endoscope itself has been reported. However, there is no clear consensus for primary repair due to the limited number of cases seen[6-8].

We report four cases in which ERCP done at tertiary referral centers induced a large duodenal perforation, which was then successfully managed with endoscopic approximation suture using multiple endoclips under transparent cap-assisted endoscopy.

CASE REPORT

Case 1

An 80-year-old woman with Klatskin tumor underwent ERCP. Three days later after endoscopic biliary drainage and biopsy, ERCP was attempted for the placement of the metallic stent. During the procedure, the duodenoscope slipped into the duodenum and caused an approximately 13 mm-sized perforation in the duodenal bulb. After early recognition of perforation, an immediate attempt to seal the perforation with 8 endoclips (Endoclip HX-600-090L; Olympus Optical Co., Ltd., Tokyo, Japan) was made successfully under transparent cap-assisted endoscopy (Figure 1A and E). Following the endotherapy, placement of percutaneous transhepatic biliary drainage (PTBD), peripheral parenteral nutrition, intravenous high-dose proton pump inhibitor (PPI), and broad-spectrum antibiotics (3rd generation cephalosporin + metronidazole) was initiated. A nasoduodenal tube was not placed. An abdominal X-ray showed a pneumoperitoneum in the subphrenic area (Figure 2A). However, she remained symptom-free without additional complications. Six days later, a contrast passage *via* endoscope showed no evidence of leakiness and the pneumoperitoneum was completely resolved (Figure 2B). She resumed a scheduled diet 6 d after the clipping and was discharged on day 12 (Tables 1 and 2).

Case 2

A 72-year-old woman with a history of left hepatic lobectomy and cholecystectomy 11 years prior to the procedure underwent ERCP for the segmental stricture of right intrahepatic duct and stones after PTBD. During the insertion of the duodenoscope into the stomach, severe rigidity was felt and the duodenoscope suddenly slipped into the duodenum. This caused an approximately 13 mm-sized perforation in the lateral wall of the second portion of the duodenum. Subsequently, an attempt to seal the perforation was successfully made with 5 endoclips under transparent cap-assisted endoscopy (Figure 1B and F). Peripheral parenteral nutrition, intravenous high-dose PPI, and broad-spectrum antibiotics were initiated. An abdominal computed tomography (CT) showed a pneumoretroperitoneum (Figure 3A). She remained symptom-free 2 d later, and did not develop any complications. A repeat abdominal CT performed 6 d later showed markedly decreased pneumoretroperitoneum and fluid collection, and there was no contrast leak on upper gastrointestinal investigations (UGIs) (Figure 3B). She resumed a normal scheduled diet 7 d after clipping and was discharged on day 10.

Case 3

A 69-year-old woman was referred due to pancreatic cancer. An abdominal CT scan revealed about a 5 cm-sized pancreatic head cancer with multiple hepatic metastases. ERCP was considered for jaundice and evaluation of the biliary system. At first, a trainee who had less than 6 mo experience inserted the duodenoscope. He tried to shorten the scope loop in the duodenum several times, but failed to shorten the scope loop due to duodenal rigidity resulting from the duodenal invasion of pancreatic cancer. The trainee then notified his supervisor, who was observing the procedure. He found about a 10 mm-sized duodenal perforation, which might have been caused by excessive shortening of the scope in the restricted lumen. Successful closure was immediately performed using 4 endoclips under transparent cap-assisted endoscopy (Figure 1C and G). The next day, the patient complained of abdominal pain, but there was no interval change except mild leukocytosis. Fasting and hydration were ordered and abdominal CT scans were taken twice, 24 h apart, but the pneumoperitoneum was not found to be progressive (Figure 4A). On the 8th day after duodenal perforation, UGIs showed no contrast leakage (Figure 4B), and she was put on a diet. After discharge from the hospital 10 d later, the patient received gemcitabine chemotherapy.

Case 4

A 63-year-old man with a history of cholecystectomy 10 years before the procedure underwent ERCP for choledocholithiasis. During insertion of the duodenoscope, an approximately 12 mm-sized perforation occurred in second portion of the duodenum secondary to trauma from difficult passage of the duodenoscope due to bulb deformity caused by recurrent duodenal ulcers. A successful attempt to seal the perforation with 5 endoclips was made under long transparent cap-assisted endoscopy (Figure 1D and H), followed by PTBD. Conservative managements were initiated as mentioned above. Simple X-ray and abdominal CT showed a pneumomediastinum, pneumoperitoneum, and subcutaneous emphysema. The patient remained symptom-free 3 d later and did not develop any complications. An abdominal CT repeated 4 d later showed markedly decreased pneumoretroperitoneum (Figure 5) and a scheduled diet was started. The remaining CBD stones were removed by ERCP 25 d later.

DISCUSSION

Duodenal perforation is an infrequent complication of ERCP. It extends beyond the intramural portion of the bile duct and is usually associated with sphincterotomy in about 1% of patients[9]. Retroperitoneal duodenal perforations represent the majority of cases and usually are

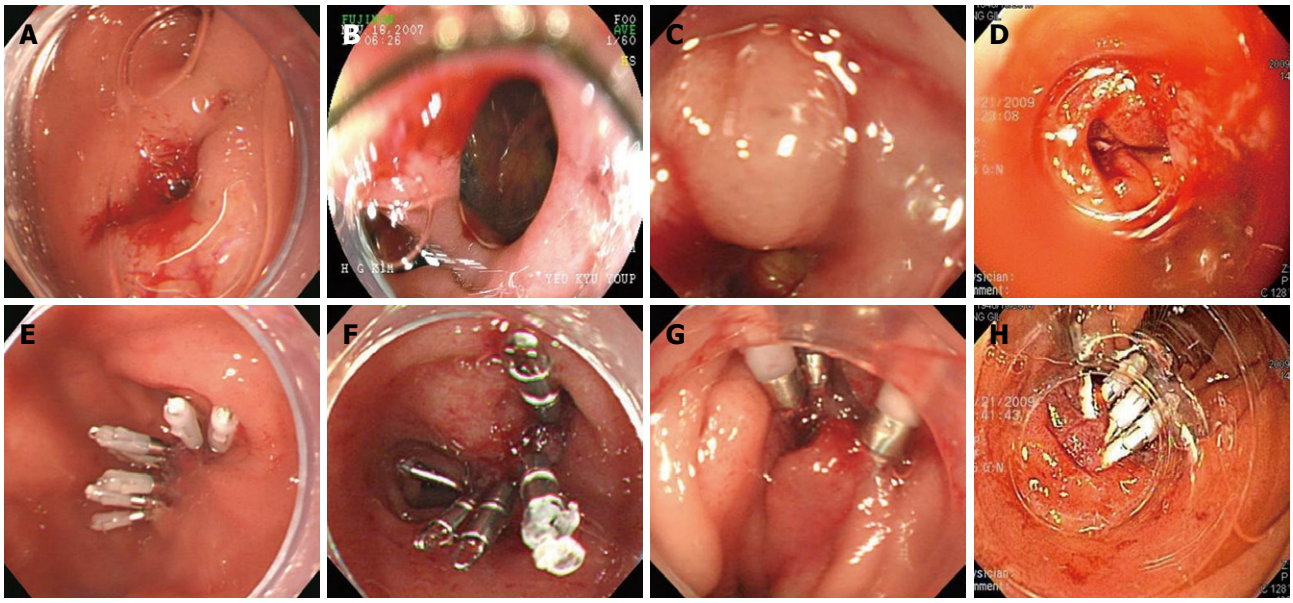


Figure 1 Endoscopic images of four cases demonstrating a large perforation on bulb and lateral wall of the second portion of duodenum, and successful primary endoscopic closure using multiple endoclips.

Table 1 Patient characteristics

Case No.	Age (yr)/sex	ERCP indication	Perforation site	Perforation size (mm)	Diagnosis	Experience of ERCPs (yr)
Case 1	80/F	Klatskin tumor	Bulb	> 10	During ERCP	< 2
Case 2	72/F	IHD stricture	2nd portion, lateral wall	> 10	During ERCP	< 2
Case 3	69/F	Jaundice	2nd portion, lateral wall	> 10	During ERCP	< 1
Case 4	63/M	Choledocholithiasis	2nd portion, lateral wall	> 10	During ERCP	< 1

ERCP: Endoscopic retrograde cholangiopancreatography; IHD: Intrahepatic duct.

Table 2 Therapeutic outcomes

Case No.	No. of endoclip	Antibiotics (d)	PPI	Gastric drainage	Biliary drainage	Start diet/ hospital stay (d)	Mortality or Cx.
Case 1	8	3rd generation cephalosporin/metronidazole (7/5)	Pantoprazole 40 mg IV	-	PTBD	6/12	-
Case 2	5	3rd generation cephalosporin/metronidazole (7/3)	Pantoprazole 40 mg IV	-	PTBD, PTCS	7/10	-
Case 3	4	3rd generation cephalosporin/metronidazole (8/5)	Pantoprazole 40 mg IV	-	PTBD	8/10	-
Case 4	5	3rd generation cephalosporin/metronidazole (14/7)	Pantoprazole 40 mg IV	-	PTBD	4/27	-

PPI: Proton pump inhibitor; PTBD: Percutaneous transhepatic biliary drainage; PTCS: Percutaneous transhepatic cholangioscopy.

due to papillotomy, whereas intraperitoneal perforations are much less common and caused by the endoscope itself[10]. Direct duodenoscope-induced perforation is much less common, accounting for 0.1% of patients who undergo ERCP, but tends to be large and further away from the ampulla[4,5,11,12]. Known risk factors of an ERCP-related perforation might include old age, suspected sphincter of Oddi dysfunction, dilated bile duct, papillary stenosis, Billroth-Ⅱ reconstruction, pre-cut sphincterotomy, and long procedure duration[5,13-15].

Immediate surgery after diagnosis is the current standard treatment for duodenal perforations caused by an endoscope. Stapfer *et al*[4] and Howard *et al*[3] have proposed a classification scheme for duodenal injury by dividing it into four and three types, respectively, according to anatomical location, mechanism of injury, and severity. Type I (lateral or medial wall duodenal perforation; Stapfer *et al*) or Group Ⅲ (duodenal perforation remote from the papilla; Howard *et al*) injuries are usually large and require immediate surgery for repair. In a study by Stapfer *et al*, surgery was recommended for patients with the following criteria: large contrast extravasation on ERCP/UGIs, contrast-enhanced CT scans showing intra- or retroperitoneal fluid collection, massive subcutaneous emphysema or suspected perforation in association with retained material (i.e. stones, ERCP wire/basket)[4]. In cases of perivaterian injuries, they suggested conservative management with serial radiographic examination. Howard *et al*[3] also suggested the use of endoscopic drainage to divert the bile, pancreatic, and/or duodenal

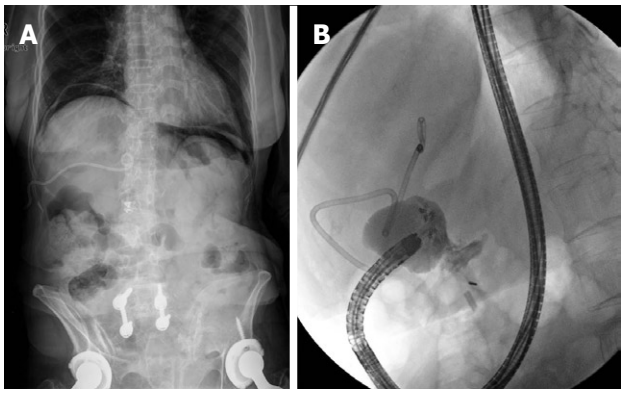


Figure 2 A simple abdomen X-ray shows both subphrenic pneumoperitoneum (A) and 6 d later, the follow-up upper gastrointestinal investigation (UGI) reveals no contrast leaks (B).

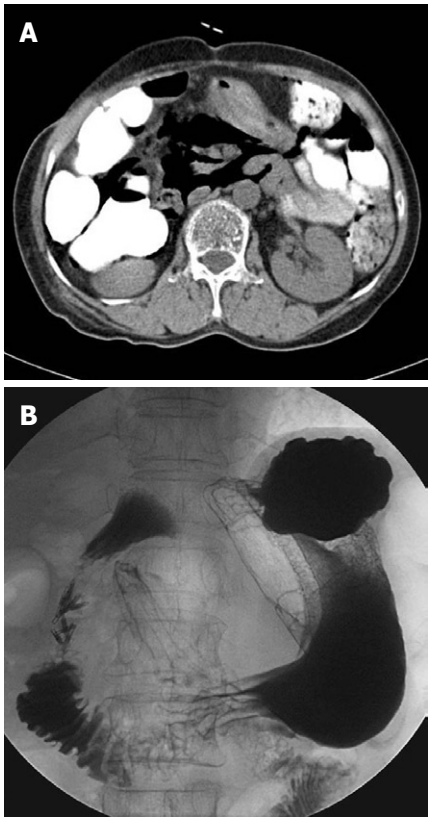


Figure 3 An abdominal computed tomography (CT) shows a severe pneumoretroperitoneum (A), and follow-up UGIs done 6 d later reveal no contrast leaks (B).

fluids away from the perforation, and showed that the endoscopic approach reduced the rates of surgery, mortality, and length of hospital stay.

However, unlike more common spontaneous perforation resulting from peptic ulcer disease, endoscopic therapy-related iatrogenic perforations have a relatively lower chance of bacterial contamination in a fasting state, and there is therefore sometimes an opportunity to manage these patients using nonsurgical means. A small amount of bacterial contamination may be controlled by the administration of antibiotics[16]. Recently, trials of endoscopic

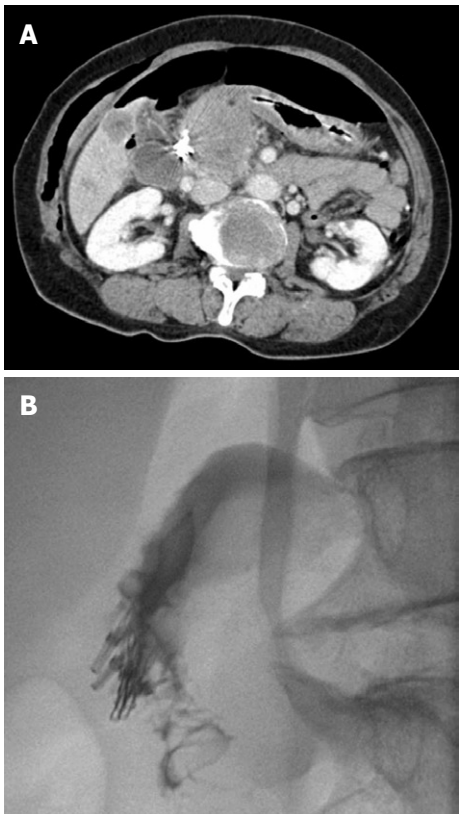


Figure 4 Initial abdominal CT following perforation shows pneumoperitoneum and subcutaneous emphysema (A), and follow up UGIs performed 8 d later reveal no contrast leaks (B).

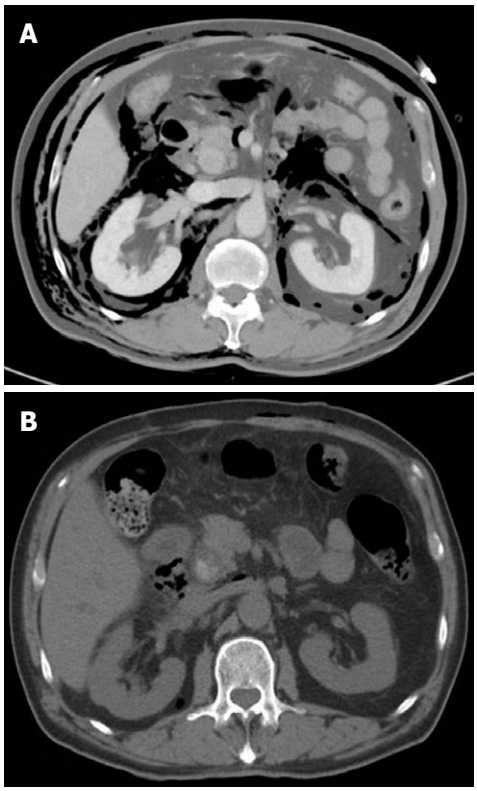


Figure 5 Abdominal CT images showing a pneumoretroperitoneum, and subcutaneous emphysema following perforation (A) and a marked improvement 4 d after conservative management (B).

management have been performed. There have been sporadic reports about the use of an endoscopic clipping device for the closure of iatrogenic perforations during endoscopic mucosal resection (EMR) or sphincterotomy in the esophagus, stomach, and duodenum[17-19]. Though surgery remains the standard treatment for duodenal perforations caused by the endoscope itself, the outcomes from case reports support the beneficial role of endoclips in the closure of these defects[6-8,20]. In particular, some reports described that nonsurgical treatment is possible for the perforation of the upper gastrointestinal tract when peritonitis remains localized. The clinician's familiarity with endoclips and their immediate availability and proper use may avoid surgery for a selected group of patients with a high surgical risk.

Kaneko *et al*[16] suggested some conditions for endoscopic repair using a clipping device in EMR-related perforation. Their suggestions included prior preparation of the patients, quick recognition of perforation, the diameter of perforation being less than the width of the clip's nail, the shape of the opening must be smooth and suitable for drawing the edges together, and an excellent visual field. In endoscopic management, quick recognition and rapid endoscopic closure were the keys to success in limiting the degree of peritoneal contamination and pneumoperitoneum, as delayed diagnosis and surgery are associated with a high mortality rate[4,21]. However, nonsurgical suturing therapy using endoclips is not yet widely accepted as the primary management of ERCP-related duodenal perforation.

In the four cases presented here, the experience of endoscopist and patient old age may be risk factors. All the perforations were done by inexperienced endoscopists who only had one or two years of therapeutic ERCP experiences. However, routine surgery was not required in all patients. The endoscopists could detect the injury early, the visual field was relatively clear, and the endoscopic manipulation was performed in minimal time in all cases. These were the reasons why the primary closure was successful despite a large perforation of more than 10 mm. The perforation was detected very early during ERCP because it occurred in the course of duodenoscope insertion. Cap-assisted endoscopy method under direct vision through a transparent hood was also helpful in reducing the manipulation time of the procedure by allowing a good visual field and ensuring the safety margin during clipping. The cap can facilitate the displacement of any mucosal folds that obscure the lumen and is very useful for overcoming the sharp angulations[22].

Conservative treatment includes giving the patients nothing by mouth, broad-spectrum antibiotics, PPI, and diversion of the bile and pancreatic secretion, or nasogastric or nasoduodenal decompression. However, there were differences in the follow-up method and interval, duration of conservative management methods (fasting, PPI, and antibiotics), and the time when a normal diet was started. Following immediate closure, the use and duration of broad-spectrum antibiotics or PPI was not clear. In our cases, routine nasogastric or duodenal drainage was not used because of early successful closure and biliary diversion by PTBD. Therefore, we think that these procedures are not always required in such cases. Normal diet should be resumed after confirming the complete closure of the wound by UGIs. If patients don't have any clinical symptoms and contrast leakage, earlier resumption of normal diet may be possible.

In summary, primary approximation closure using endoclips under cap-assisted endoscopy of iatrogenic duodenal perforation during ERCP was a safe and feasible technique for even a large free wall perforation. Although the surgical operation remains the standard treatment for duodenal perforation, these cases support the use of endoscopic closure of the perforation with conservative treatments for selected cases of the injury caused by the endoscope itself.

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CASE REPORT

Living donor liver transplantation for an adult patient with situs inversus totalis

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Abstract

This recipient with situs inversus totalis (SIT) was a 60-year-old female who had hepatitis B-related end-stage liver disease. Preoperative donor evaluation showed that the right posterior section satisfied graft volume and was space-fitting in the recipient hepatic fossa when it was rotated 180 degrees. The operation and postoperative course progressed satisfactorily. Three weeks after living donor liver transplantation (LDLT), the graft function was disturbed by compression of bottom-placed right hepatic vein. This was treated with a vascular stent and subsequently the graft function was normalized. The present case shows that LDLT for patients with SIT using a right posterior section graft is feasible.

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Key words: Living donor liver transplantation; Situs inversus totalis; Right posterior section graft

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INTRODUCTION

Situs inversus totalis (SIT) is a rare congenital anomaly with complete reversal of all visceral organs. It occurs once in about 15 000 to 20 000 births and has unknown cause. Sometimes SIT is associated with other anomalies such as biliary atresia, visceral vascular anomaly, and polysplenia. In general, most people with SIT have no complications and perform normal physical activities. However, this condition could be a serious problem when he/she needs liver transplantation because the liver has asymmetrical anatomy that causes technical difficulties in graft positioning and vascular anastomoses in the SIT recipient. For this reason, it has been regarded as a contraindication to liver transplantation.

There are some technical reports of whole graft liver transplantation for adult SIT recipients[1-3]. Also, cases of living donor liver transplantation (LDLT) for the adult patient with SIT have been reported using the left or right lobe with midline positioning of the graft[4,5]. However, midline positioning in the SIT recipient may present two major concerns. Firstly, graft displacement due to redundant space of recipient's hepatic fossa could result in vascular torsion and kinking; and secondly, the recipient's inferior vena cava could be compressed by the graft. To overcome these problems, we performed reversed orthotopic implantation using right posterior section, Couinaud segment VI + VII, grafted into the recipient's hepatic fossa[6].

CASE REPORT

Our recipient was a 60-year-old female with advanced liver cirrhosis caused by hepatitis B virus. Her body weight was 60 kg and the Model for End-stage Liver Disease (MELD) score was 19. The donor was her nephew, a 45-year-old male with a suitable volume of right posterior sector on preoperative computed tomography (CT) scan. Preoperative CT scan showed hilar trifurcation of portal vein and measurement of total liver volume (TLV) was 1100 mL. The right liver volume of the donor was 780 mL (71% of TLV), the left lobe volume was 320 mL, and the right posterior section volume was 510 mL (46.4% of TLV). A right posterior section graft was the only suitable type of donor hepatectomy to ensure donor safety and appropriate volume of graft to the recipient. Preoperative measurement of the right posterior section graft to recipient weight ratio (GRWR) was 0.85%. Preoperative simulation showed that the donor's right posterior section fit well into the recipient's left subphrenic space with 180-degree flip-over (facing backward) implantation method.

The donor operation was performed uneventfully according to the usual procedure of right posterior sectionectomy including right hepatic vein (RHV). There was no significant inferior right hepatic vein in the graft. The actual graft weight was 470 g. On the back table, protruded liver tissue behind the right posterior portal vein and RHV was cut out with an ultrasonic dissector to expose the portal and hepatic veins in reversed graft position. Under laparotomy of the recipient with inverted T-incision, complete rotation of all internal organs along the midline was observed without any vascular anomaly. Recipient hepatectomy was not complicated. The anhepatic time was 45 min. The reversed right posterior section graft was located in the recipient's left subphrenic space. To gain sufficient graft outflow, a piece of cryopreserved vein patch was applied on the ventral portion of the graft's RHV and a modified piggyback anastomosis was done. Vascular anastomoses of the portal vein and hepatic artery were performed as usual. The cold ischemic time was 2 h and the warm ischemic time was 45 min. In the reversed position of the right posterior section graft, the bile duct was located behind the portal vein. Thus, temporary downward traction of the portal vein was necessary for biliary anastomosis (Figure 1). Biliary anastomosis between the recipient's common hepatic duct and the graft's duct was performed successfully with 7-0 prolene continuous suture. Splenectomy was performed according to our indication of thrombocytopenia and splenomegaly. Total operation time was 11 h and 35 min.

The patient's postoperative course was excellent for 3 wk after transplantation, but the serum levels of liver enzymes subsequently started to elevate. However, there was no evidence of acute rejection on needle biopsy. Abdominal CT scan and hepatic Doppler ultrasound showed disturbance of blood flow in segment 6 RHV (V6 RHV) due to compression by the graft itself. A long endovascular stent was inserted into V6 RHV and the liver recovered quickly (Figure 2). Consequently, the patient was discharged at postoperative day 45 with good liver function. She has maintained good general condition and liver function for 8 mo postoperatively, up to the present time.

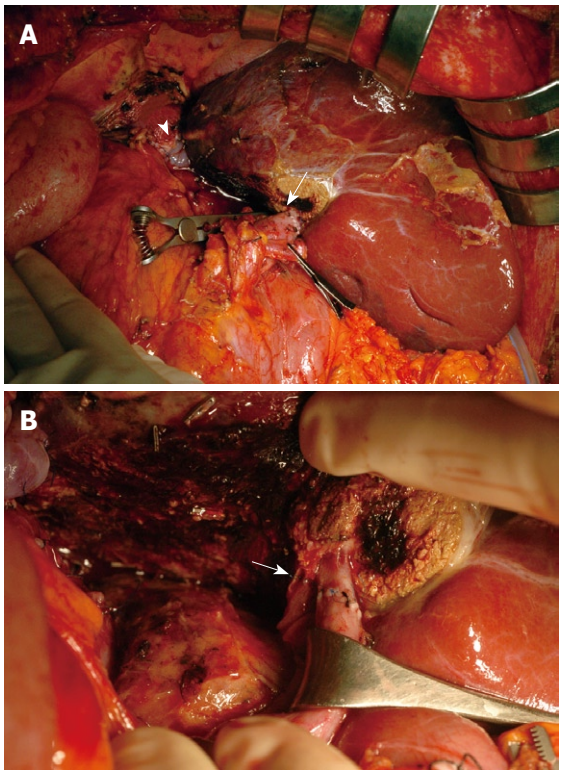


Figure 1 Reversed implantation of the right posterior section graft. A: Modified piggyback anastomosis of right hepatic vein (arrow head) and portal vein anastomosis (arrow); B: Duct opening of the graft (arrow).

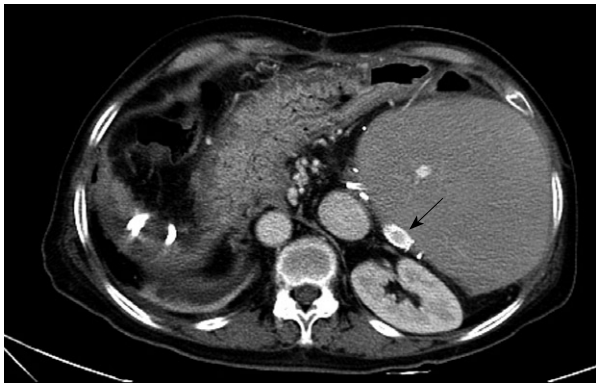


Figure 2 Postoperative 6 mo computed tomography scan showing good graft positioning and patent endovascular stent in the right hepatic vein (arrow).

DISCUSSION

The major technical problem of liver transplantation for adult SIT is the spatial mismatch between the graft's and recipient's left upper quadrant space. Liver transplantation for the SIT patient has been performed at several centers, mainly using techniques of midline positioning or left shifting with/without clockwise rotation of the graft.

However, these techniques have possible complications, such as graft displacement due to unfavorable position and vena cava compression by large right lobe of the graft.

Recently, Rayhill *et al*[7] reported a novel technique of 180-degree flip-over (facing backward) implantation of the whole liver graft into the recipient's left subphrenic space. They reported excellent outcome without complication. Their technique provided not only orthotopic implantation of the whole graft but also good arrangement of portal vein, hepatic artery, and bile duct. We agree that orthotopic reversed graft implantation could overcome the possible complications of midline graft positioning for the SIT recipient.

Regarding LDLT, Chun *et al*[8] reported orthotopic implantation of a right lobe graft with 180-degree flip-over method from a living SIT donor to a recipient with normal anatomy. This technique is similar to ours in reversing the graft, but when using a reversed right lobe graft, the hilar plate is located more ventrally due to voluminous anterior sector behind the hilum. This could result in shortage of portal vein and bile duct, which leads to unavoidable tension in the anastomoses, and consequent vascular stretching or kinking. To avoid such complications, Chun *et al* used a special technique to achieve sufficient length of recipient's portal vein and bile duct.

In our case, the location of the hilar plate of the right posterior sector was almost at the same level within the SIT recipient's hepatic fossa in the reversed position. This allowed us to perform easy vascular and duct-to-duct biliary anastomoses without any of the complications mentioned above. To our knowledge, this is the first report of liver transplantation using a right posterior section graft for the adult patient with SIT. According to our experience, the only disadvantage of this technique using right posterior section graft is the possible compression of the V6 RHV, located at the bottom, by the graft itself during rapid liver regeneration after LDLT. The compression of the bottom-located RHV in reverse positioning of the right posterior section graft would be a common phenomenon, because there is little space to expand behind the RHV during rapid liver regeneration. Thus, we suggest that the surgeon should be alert to detect compression of the RHV during the early postoperative period. However, this complication could easily treated by inserting an endovascular stent.

In conclusion, reversed implantation of the right posterior section graft is one good option for liver transplantation in the adult recipient with SIT. This procedure provides not only orthotopic transplantation to the SIT recipient but also convenient vascular and duct-to-duct biliary anastomoses. In using this technique, however, we have to be alert during the postoperative course because the bottom-located RHV could be compressed by the graft.

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CASE REPORT

Radiological diagnosis of duodenocaval fistula: A case report and literature review

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Abstract

Duodenocaval fistula (DCF) is an uncommon but lethal clinical entity. The high mortality has been attributed to the difficulty of diagnosis before attempts at definitive therapy. In this case report, we describe a patient with a series of computed tomography (CT) examinations over a 2-mo period in hospital. A low-density air bubble appeared in the inferior vena cava (IVC) on the second day in hospital and became clear on day 19, and gradually enlarged. Magnetic resonance imaging (MRI) also clearly demonstrated a high-signal enteric contrast medium or thrombus and signal-void air bubbles in the IVC. However, cavography did not show the filling defect. We suggest that noninvasive CT and MRI should be chosen as a first-line investigation, and IVC, including the surrounding structures, should be carefully reviewed on images if DCF is clinically considered.

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Key words: Duodenocaval fistula; Computed tomography; Magnetic resonance imaging

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INTRODUCTION

Duodenocaval fistula (DCF) is an uncommon but lethal clinical entity that has previously been reported in only 39 patients in the English-language literature[1-9]. The high mortality of 41% (16/39) has been attributed to the difficulty of diagnosis before attempts at definitive therapy. We present a case of DCF that underwent a series of endoscopy, computed tomography (CT), magnetic resonance imaging (MRI), and cavography examinations, and a review of the literature with respect to radiological manifestation of DCF.

CASE REPORT

A 78-year-old man was hospitalized because of right-sided abdominal pain for 1 mo, associated with fever, rigors, and chills for 1 d. His medical history was significant for right nephrectomy 5 years ago because of renal cell carcinoma. A mass was found in the right adrenal gland and gamma knife radiotherapy was performed 10 mo ago. On physical examination, his initial vital signs were temperature 39.5℃, heart rate 120 beats/min, blood pressure 110/70 mmHg, respiration 25 breaths/min, and SaO_2 97%. Initial laboratory studies showed a leukocyte count of 22 × 10^9/L, hemoglobin 80 g/L, platelet count 33 × 10^9/L, and normal blood chemistry, clotting parameters, and liver function tests. Blood cultures revealed *Enterococcus faecium* and *Enterobacter cloacae* bacteremia and *Candidia albicans* fungemia. Treatment with intravenous broad-spectrum antibiotics and antifungal

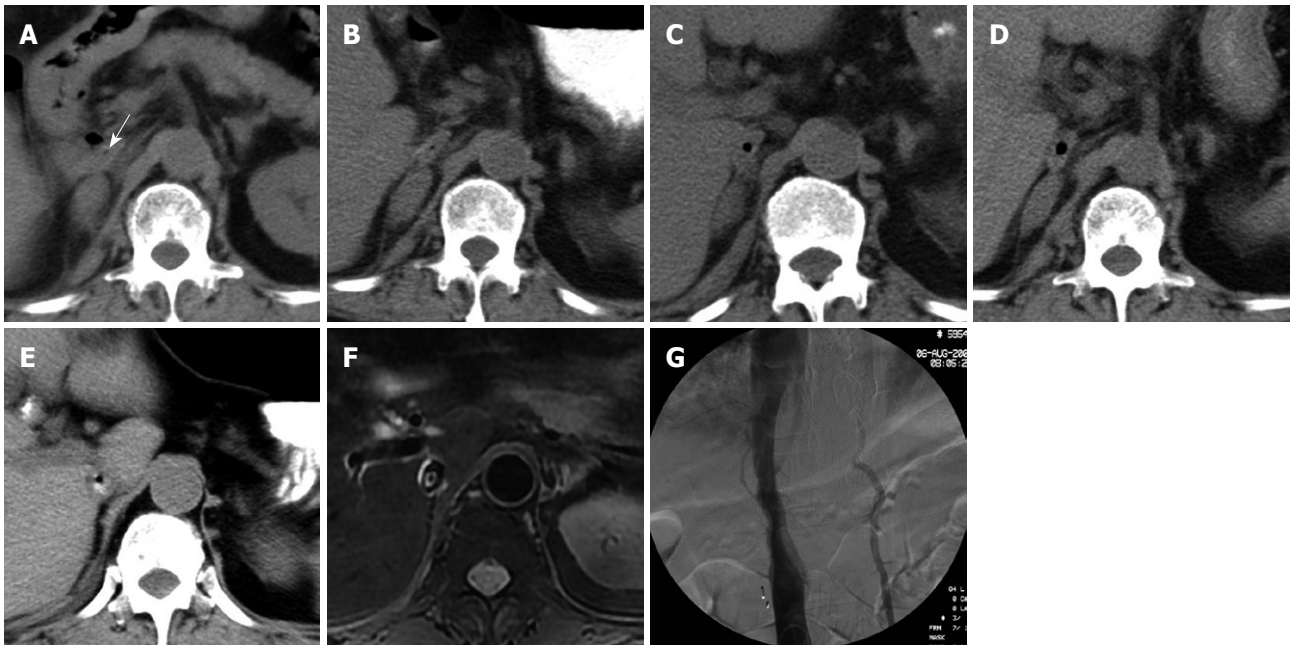


Figure 1 During the 2-mo hospital stay, a small, low-density air bubble appeared in the inferior vena cava (IVC) and gradually enlarged on follow-up computed tomography (CT) scans. Enteric contrast medium leaked into the IVC. Magnetic resonance imaging (MRI) clearly demonstrated the high-signal enteric contrast medium or thrombus and signal-void air bubbles. Cavography did not reveal the thrombus in the IVC. A: CT scan on the second hospital day. A low-density dot loomed up in the IVC (arrow). A mass in the right side adrenal gland was also seen; B: CT scan on the 19th hospital day. The low-density dot became more evident; C, D: CT scan on the 59th and 67th hospital day. The low-density dot (air bubble) was gradually enlarged; E: CT scan on the 73rd hospital day with water-soluble enteric contrast demonstrated air bubble surrounded by contrast medium in the IVC; F: MRI T2WI on the 74th hospital day demonstrated the high signal enteric contrast medium or thrombus and signal void air bubble in the IVC; G: Inferior vena cavogram on the 75th hospital day showed no evident thrombus in the IVC.

therapy (meropenem and caspofungin) resulted in some improvement. However, during the 76 d in hospital, the patient developed intermittent fever, high leukocyte count, sepsis and fungemia. About 1 mo after admission, the patient had watery stools, which were positive for occult blood. A series of upper gastrointestinal (GI) endoscopy examinations, abdominal ultrasound, CT, MRI and inferior vena cavography were performed during the hospital stay. Upper GI endoscopy (on day 3 in hospital) revealed a giant ulcer in the second portion of the duodenum, but no active bleeding. Abdominal ultrasound found nothing except a mass in the right adrenal gland. A series of abdominal CT scans were performed on days 2, 19, 59, 67 and 73 in hospital (Figure 1). Low-density air bubbles were incidentally found in the inferior vena cava (IVC) (at the level of the first lumbar vertebra, adjacent to the duodenum) by CT on day 67. A review of the previous CT scans showed a low-density dot in the IVC on the second day in hospital, which became clear on day 19, and gradually enlarged. A repeat abdominal CT scan (day 73) with water-soluble enteric contrast demonstrated low-density air bubbles surrounded by high-density contrast medium or thrombus in the IVC, which suggested DCF. T2-weighted MRI (day 74th day of hospitalization, Figure 1) revealed high-signal enteric contrast medium or thrombus and signal-void air bubbles in the IVC. However, inferior vena cavography (day 75 of hospitalization) showed no evidence of IVC thrombus (Figure 1). Surgical consultation was scheduled but before it could be performed, the patient had an episode of hematemesis. Massive hemorrhage ensued and the patient died despite aggressive resuscitation efforts. Postmortem examination revealed a fistula between the second portion of the duodenum and the IVC.

DISCUSSION

DCF is a rare occurrence that typically arises as a complication from migrating IVC filters, peptic ulcer disease related to retroperitoneal tumor resection in association with radiation therapy, or transmural migration of ingested foreign bodies. Diagnosis of this entity is challenging because of the nonspecific nature of the symptoms and is rarely made before laparotomy or autopsy. The most common presentations of DCF are sepsis and GI hemorrhage. Sepsis is generally polymicrobial in origin, and caused by Gram-positive and Gram-negative enteric bacteria in the systemic circulation. Fungemia may also occur, as in the present case[1-9]. Endoscopy typically discloses a duodenal ulcer that may show visible bleeding, but the extent of penetration of the ulcer is often underappreciated[1]. CT provides noninvasive evaluation of the IVC and the adjacent structures. It is capable of detecting thrombus and air bubbles in the IVC, infectious fluid collection or abscess around the IVC and duodenum, incarcerated foreign bodies, and migrated caval filters[1-4]. CT correctly can identify DCF in approximately 50% of patients[2]. In this case, a low-density dot appeared in the IVC on the second day in hospital, became clear on day 19, and gradually enlarged over the 2-mo hospital stay. Air bubbles in the IVC may have been produced by bacteria or pushed in by gut peristalsis. Repeated CT

examinations are necessary when air bubbles are too small to make a diagnosis. For detecting thrombus in the IVC, enhanced CT is essential. MRI was not used for the diagnosis of DCF in previous studies. With conventional or flow-sensitive sequences, MRI can clearly delineate thrombus in the IVC, even without intravenous contrast medium[10,11]. Thrombus, intestinal contents and enteric contrast show abnormal signals against signal-void blood flow in the IVC. In the present case, MRI clearly demonstrated high-signal enteric contrast medium or thrombus and signal-void air bubbles in the IVC. Cavography has revealed thrombus or filling defects in the IVC, but was diagnostic of DCF in only two out of six cases[1,4,12]. In the present case, cavography did not show enteric contrast medium or thrombus in the IVC. The most likely reason was that the thrombus was flushed out by the high-pressure injection of intravenous contrast medium. Fatal pulmonary embolization composed of intestinal contents through a DCF has been reported[13]. Our patient died of hematemesis 24 h after cavography. Therefore, care should be taken to perform invasive cavography to detect unstable thrombus of DCF in the IVC.

In summary, we report this case to remind clinicians that noninvasive CT and MRI should be chosen as a first-line investigation for diagnosis of DCF. If DCF is clinically considered, IVC and the surrounding structures should be carefully reviewed by imaging.

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SCIENTOMETRICS

Role of *Orvosi Hetilap* in the development of Hungarian gastroenterology

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Abstract

AIM: To analyze the contribution of *Orvosi Hetilap* (Hungarian Medical Journal) to the field of gastroenterology.

METHODS: All issues of the journal between 1857 and 2008 and identified original articles and reviews dealing with gastroenterology were reviewed. The rate of publications, the thematic distribution and foreign sources of knowledge were assessed. The dates that major achievements in gastroenterology were introduced in Hungary were compared to those dates in Western medicine.

RESULTS: A total of 4799 original/research articles on gastroenterology were published, which represents 11.1% of the total publications. Thematic rankings showed that liver and biliary diseases represented 20.36% of the total, followed by gastric diseases (9.35%) and surgery (8.77%). A total of 268 foreign journals were reviewed: 50.9% were German, 30.4% English, 12.1% French and only 6.6% were in other languages. The major achievements of gastroenterology were introduced with varying delays compared to Western countries.

CONCLUSION: *Orvosi Hetilap* has made a large contribution to the development of Hungarian gastroenterology. The high proportion of gastroenterology studies underlines the importance of digestive diseases in public health.

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Key words: Content analysis; Gastroenterology; Hepatology; *Orvosi Hetilap*; Scientometrics

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INTRODUCTION

Nineteenth century Europe witnessed the publication of national medical journals. In Hungary, *Orvosi Hetilap* (*OH*; Hungarian Medical Journal) was first published in 1857, thus making it one of the first medical journals worldwide (Table 1). The importance of the journal in the development of Hungarian medical science and medical language is unrivalled, a fact that has been recognized many times. Much less is known, however, about the role of *OH* in the development of special fields, such as Hungarian gastroenterology. Medical historians divide the past 150 years into six periods according to the editors-in-chief of *OH* (Table 2)[1]. Most of the editors were eminent figures in Hungarian medicine, and were quoted in foreign textbooks of medical history. To the best of my knowledge, this is the first complete content analysis of a medical journal in the field of gastroenterology.

The aim of the study was to track the timeline and make a thematic analysis of the papers dealing with diseases of the gastrointestinal tract, as published in *OH* between 1857 and 2008.

Table 1 Publishing timeline of the national medical journals

1798: *Journal de Medécine*	Paris
1809: *Journal of the Royal Society of Medicine*	London
1812: *New England Journal of Medicine*	Boston
1823: *The Lancet*	London
1840: *British Medical Journal*	London
1850: *Wiener Medizinische Wochenschrift*	Vienna
1854: *Nederlander Tijdschrift der Geeneskunde*	Amsterdam-Utrecht
1857: *Orvosi Hetilap*	Budapest
1875: *Deutsche Medizinische Wochenschrift*	Stuttgart
1883: *Journal of American Medical Association*	Chicago
1893: *La Presse Médicale*	Paris

Table 2 Editorial periods of *OH*

Period	Editor-in-chief	Specialty	Duration (yr)
1857-1888	L. Markusovszky (1815-1893)	Public health	32
1889-1905	E. Hőgyes (1847-1906)	Bacteriologist, pharmacologist	16
1905-1922	M. Lenhossék (1863-1937)	Anatomist	17
1923-1944	Z. Vámossy (1868-1953)	Pharmacologist	21
1948-1989	T. Trencséni (1907-1996)	Internist	41
1989-2008	J. Fehér	Gastroenterologist, hepatologist	20

OH: *Orvosi Hetilap*.

Table 3 Rate of gastroenterology publications in *OH* between 1857 and 2008 *n* (%)

Editorial period	Total original articles	Gastroenterology articles	Total journal reviews	Gastroenterology article reviews
1857-1888	3416	312 (9.1)	1386	126 (9.0)
1888-1904	2102	143 (6.8)	435	39 (8.9)
1905-1922	2906	406 (13.9)	7332	658 (8.9)
1923-1944	7757	696 (8.9)	11586	937 (8.0)
1948-1989	19583	2175 (11.1)	31489	3965 (12.5)
1989-2008	7682	1067 (13.8)	19575	1698 (8.6)
Total No.	43446	4799 (11.1)	71803	7423 (10.3)

MATERIALS AND METHODS

I manually reviewed the journal volumes between 1857 and 2008. The full papers (both original research and reviews) as well as foreign journal article reviews were identified and classified according to their subjects and origin. All gastrointestinal, liver, biliary tract and pancreatic diseases were included. Articles on epidemics (cholera and typhus) that involved the digestive tract were excluded, being more the subject of epidemiology and microbiology, as considered by medical historians[2]. The thematic analysis of the articles and journal reviews was performed using specific key words that occurred in the titles. The data were entered into an Excel database. The differences between the editorial periods were assessed using analysis of variance and the Kruskal-Wallis test, with a significance level of $P = 0.05$. The statistical work was performed using Statsoft Inc. version 9.0 software (Tulsa, OK, USA). The dates when some major achievements in gastroenterology were introduced in Hungary were compared to those for Western medicine.

Table 4 Thematic breakdown of full papers published in *OH* between 1857 and 2008[1] *n* (%)

Subject	Articles
Anatomy	18 (0.30)
Pathology	121 (2.02)
Physiology	132 (2.20)
Esophagus	194 (3.24)
Stomach	559 (9.65)
Small bowel	298 (4.98)
Appendix	93 (1.55)
Large bowel	268 (4.63)
Liver	871 (14.57)
Biliary tract	346 (5.79)
Pancreas	354 (5.92)
Peritoneum	102 (1.70)
Tumors	195 (3.26)
Surgery	524 (8.92)
Radiology	165 (2.76)
Endoscopy	246 (4.11)
Ultrasound	69 (1.25)
Pediatrics	185 (3.09)
Laboratory medicine	145 (2.42)
Peptic ulcer	189 (3.16)
Inflammatory bowel diseases	102 (1.70)
Viral hepatitis	219 (3.96)
Infectious diseases (non-epidemic)	200 (3.34)
Laparoscopic surgery	27 (0.45)
Transplantation	39 (0.67)
Genetics	61 (1.02)
General topics	252 (4.28)
Total	5974 (100)

[1]Some articles are included more than once, based on keywords in the title.

RESULTS

General data

The volume of *OH* gradually increased from about 400 pages a year at the outset to 800 pages at the turn of the century, before increasing further. During World Wars I and II, both the extent and the number of publications decreased. Between 1944 and 1948, the publication of *OH* was interrupted because of the post-war economic depression (mainly due to lack of paper and printing facilities). After 1948, the volume averaged out at 2469 pages/year, which reflected the increasing number of original articles, journals and book reviews.

Full papers (original research and reviews)

A total of 43446 papers were published during the period studied (Table 3). While the editorial periods were unequal, the number of papers/year was calculated and it increased from 36 (1857-1904) to 64 (1905-1922) ($P = 0.0002$), before changing to 60 (1923-1944) ($P = 0.46$), 89 (1948-1989) ($P = 0.0001$) and 53 (1989-2008) ($P = 0.48$). For the whole period, gastroenterology articles represented 11.1% (6.8-13.9) of the total number of papers published in *OH*. The thematic breakdown of full papers on gastroenterology is shown in Table 4.

Table 5 Introduction of some major gastroenterology achievements in Western countries and Hungary[2-6]

Achievement	International application (year, author, location)	Hungarian application (year, author, location)
Rigid gastroscopy	1868 (A. Kussmaul, Freiburg)	1902 (J. Zimmermann, Budapest)
Gastric resection	1880 (L. Rydiger, Vienna)	1900 (M. Herczel, Budapest)
Radiography	1895 (K. Roentgen, Würzburg)	1896 (J. Klupathy, Budapest)
Cholecystectomy	1881 (C. Langenbuch, Berlin)	1889 (D. Velits, Budapest)
Liver biopsy	1938 (I. Silverman, New York)	1948 (L. Friedrich, Budapest)
Vagotomy	1943 (L. Dragstedt, Chicago)	1948 (E. Hedri, Budapest)
B-mode abdominal ultrasound	1957 (I. McDonald, London)	1973 (Á. Szebeni, Budapest)
Fiberoptic gastroscopy	1958 (B. Hirschowitz, Birmingham, USA)	1965 (T. Jávor, Debrecen)
Fiberoptic colonoscopy	1970 (F. Matsunaga, Tokyo)	1972 (L. Simon, Jászberény)
Endoscopic retrograde cholangio-pancreatography	1970 (I. Oi, Tokyo)	1971 (L. Sáfrány, Budapest)
Endoscopic sphincterotomy	1973 (M. Classen, Munich)	1976 (J. Papp, Budapest)
Liver transplantation	1967 (T. Starzl, Denver)	1995 F. Perner, Budapest)
Laparoscopic cholecystectomy	1985 (T. Mühe, Bobelein)	1990 (I. Kiss, Pécs)
Capsule endoscopy	2000 (P. Swain, London)	2002 (I. Rácz, Győr)

Table 6 Profile of foreign journal reviews in *OH* between 1857 and 2008

Editorial period	No. of articles reviewed	No. of journals	English (%)	German (%)	French (%)	Other Languages (%)
1857-1888	211	86	17.9	52.5	15.9	13.2
1888-1904	381	113	12.6	64.5	17.7	5.2
1905-1922	658	112	15.6	62.2	14.6	7.6
1922-1944	936	121	19.6	57.1	16.9	6.4
1948-1989	3965	178	52.4	38.4	2.9	6.3
1989-2008	1698	113	64.4	31.0	2.9	0.9
Total	7849	268	30.4	50.9	12.1	6.6

Table 7 Ranking of core journals reviewed in *OH* between 1857 and 2008 *n* (%)

Journal	Articles reviewed
Deutsche Medizinische Wochenschrift	1147 (31.2)
Lancet	584 (15.9)
British Medical Journal	410 (11.2)
New England Journal of Medicine	313 (8.5)
American Journal of Roentgenology	263 (7.1)
Zentralblatt für Chirurgie	227 (6.1)
Radiology	204 (5.5)
Schweizerische Medizinische Wochenschrift	194 (5.2)
Journal of American Medical Association	167 (4.5)
Münchener Medizinische Wochenschrift	157 (4.2)
Total	3666 (100)

The dates when some major achievements in gastroenterology were first introduced in Hungary were compared with their first application in Western countries (Table 5)[2-7].

Journal article reviews

Foreign medical journal reviews appeared in the pages of *OH* from the very beginning. Two hundred and sixty-eight different journals were reviewed. Between 1948 and 1959, however, their publication was interrupted for political reasons. The number and language breakdown of the journals in the editorial periods are given in Table 6.

The 10 most reviewed core journals are listed in Table 7. Overall, 3666 articles were reviewed in these journals, which represented 9.7% of the total reviews. The six English journals (60%) had 1941 articles reviewed (52.5%), while the four German journals had 1725 articles reviewed (47.5%).

DISCUSSION

During the 150 years of its publication, *OH* has covered all aspects of developing gastroenterology, from basic sciences (anatomy, physiology and pathology) to the latest achievements (abdominal imaging, laparoscopic surgery, transplantation and genetics) (Table 5). Published every week, it became the main source of professional knowledge for Hungarian physicians. The 11.7% ratio of papers dealing with diseases of the gastrointestinal tract underlines their importance in public health. Diseases of the liver and biliary tract (20.36%) and the stomach (9.35%) (especially peptic ulcers, 3.16%) were studied in most detail, although there were considerable fluctuations in the publication rates. Content analysis revealed that milestone developments of gastroenterology, as quoted, mentioned or even canonized by leading historians[3-8], were introduced in Hungary with varying degrees of delay.

A large variety of foreign journals were reviewed. An analysis of journal reviews reveals that until the 1960s, German literature was the main source of information. The reasons for this are rooted in history, given that the country was part of the Austro-Hungarian monarchy. From the 1960s onwards, British and American journals gradually became the dominant source of information until the dawn of the internet. Even so, the German weekly *Deutsche Medizinische Wochenschrift* was and remains the most frequently reviewed journal. For a closer look at the foreign sources, it would have been better to conduct a citation analysis of the full articles. However, accurate reference lists only appeared after the 1920s. Until then, references were mentioned only as footnotes, indi-

cating the name of the authors; therefore, the impact of major advances and seminal articles on the Hungarian literature cannot be calculated.

In spite of its longevity and indexing in Medline/PubMed, Index Medicus, Embase, Index Copernicus and Google Scholar, *OH* has not been assigned an impact factor. To overcome this, the current Editor-in-Chief, J. Fehér, introduced a 2-monthly English version of *OH* called the *Hungarian Medical Journal*, which has published selected articles of the parent journal since 2007. Another alternative would be the bilingual (Hungarian-English) publication of the high-quality articles. *OH* has been accessible on the internet (http://www.akademiaikiado.hu) since 2008.

The complete archive of *OH* is available in just a few university and hospital libraries. These collections have not benefited from the passing of time. In the future, the digitalization of the journal is vital to save its valuable scientific content for future generations.

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COMMENTS

Background

The development of a medical specialty can be followed by the analysis of articles, journals and book reviews published over time in a given field.

Innovations and breakthroughs

The paper is believed to be the first content analysis of the complete edition of a time-honored medical journal, *Orvosi Hetilap* (Hungarian Medical Journal; 1857-2008).

Applications

The author overviews the development of gastroenterology in Hungary over 150 years by analyzing and classifying the original articles, journal and book reviews in the field of gastroenterology, covering both basic (anatomy, pathology and physiology) and applied sciences (esophageal, gastric, intestinal, liver and biliary diseases, and diagnostic procedures), with an emphasis on the main achievements as they have occurred in the literature.

Peer review

This is an interesting historical review of 150 years of gastroenterology articles published in a weekly Hungarian medical journal.

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Comments on the article about the treatment of peripancreatic infection

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Abstract

We read with great interest the article by Tang *et al* published in issue 4 of *World Journal of Gastroenterology* 2010. The results of their study indicate that percutaneous catheter drainage in combination with choledochoscope-guided debridement is a simple, safe and reliable treatment procedure for peripancreatic infections secondary to severe acute pancreatitis. However, there are some points that need to be addressed, including data about the patients in the study and their clinical characteristics, data about infection and superinfection during the treatment and type of treatment of patients with acute necrotizing pancreatitis.

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Key words: Acute pancreatitis; Pancreatic necrosis; Infection; Non-surgical management

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TO THE EDITOR

We read with great interest the article by Tang *et al*[1] published in issue 4 of *World Journal of Gastroenterology* 2010. The article provides important data because it evaluated the new method used in treatment of pancreatic and peripancreatic infections secondary to severe acute pancreatitis (SAP). The results of their study indicate that percutaneous catheter drainage in combination with choledochoscope-guided debridement is a simple, safe and reliable treatment for peripancreatic infections secondary to SAP. The authors suggested that the procedure performed in their study could be used as an alternative to the conventional open-abdominal surgical or laparoscopic debridement in treatment of peripancreatic infection of early SAP patients.

However, there are some points that need to be addressed. In the section of RESULTS, the authors specified "the time from the onset of pancreatitis to drainage was 4-11 d (mean 5.3 d). Before the sinus tract was expanded, the external drainage was maintained for 3-5 d (mean 3.6 d)". Hence, practically all interventions were performed during the first two weeks from the onset of pancreatitis. However, it is known that SAP runs a biphasic course[2]. During the first 1-2 wk, there is a pro-inflammatory response, resulting in a systemic inflammatory response syndrome. It is a sterile response in which sepsis or infection hardly ever occurs. However, after the first 1-2 wk, there is a transition from a pro-inflammatory to an anti-inflammatory response. It is during this anti-inflammatory response that the patient is at risk for the translocation of intestinal flora and the development of infection of necrotic tissue and fluid collections. It is quite uncommon that all patients in the study had pancreatic infection in the first two weeks of the disease. In the article, there are no data confirming that peripancreatic necroses and collections were infected. Also, it is well known that continuous percutaneous drainage often leads to colonization of the cavity with microorganisms and results in frequent superinfection[3,4]. However the authors did not report any data about it.

The authors had impressive results comparing to other similar studies[3-5], but it is disputable if comparable patients were treated in this study since the authors did not present data about the clinical scoring and multiple organ failure of the included patients.

Finally, on the basis of our long-term experience, we believe that the disease catheter drainage of infected necrotic tissue is very poor in the beginning, irrespective of the catheter size we used. However, during the course of SAP, a transition from solid necrotic tissue to more liquid contents leads to a higher success rate of the evacuation of necrotic tissue from the cavities, regardless of the catheter size. Therefore, we consider that only conservative treatment with proper intravenous hydration and administration of proper antibiotics should be performed in beginning of the disease. Percutaneous drainage with vigorous irrigation should be considered when truly conservative treatment fails to resolve infected pancreatic necrosis. We consider that necrosectomy, including choledochoscope-guided debridement, may represent an overtreatment in beginning of the disease in these patients with usually poor general condition. However, it is difficult to discriminate between necrotic tissue and normal tissue, and a very high risk of bleeding from vessels may occur in necrotized tissue during or immediately after the intervention.

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Meetings

Events Calendar 2010

January 25-26
Tamilnadu, India
International Conference on Medical Negligence and Litigation in Medical Practice

January 25-29
Waikoloa, HI, United States
Selected Topics in Internal Medicine

January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology Conference

January 28-30
Hong Kong, China
The 1st International Congress on Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of Gastroenterology & Endoscopy Meeting

March 09-12
Brussels, Belgium
30th International Symposium on Intensive Care and Emergency Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian National Association for Study of the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on Diabetes PACD14

March 25-28
Beijing, China
The 20th Conference of the Asian Pacific Association for the Study of the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology and Hepatology Conference, EGHC 2010

April 14-17
Landover, Maryland, United States
12th World Congress of Endoscopic Surgery

April 14-18
Vienna, Austria
The International Liver Congress™ 2010

April 28-May 01
Dubrovnik, Croatia
3rd Central European Congress of surgery and the 5th Croatian Congress of Surgery

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual Meeting

May 06-08
Munich, Germany
The Power of Programming: International Conference on Developmental Origins of Health and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and Rectal Surgeons Annual Meeting

June 04-06
Chicago, IL, United States
American Society of Clinical Oncologists Annual Meeting

June 09-12
Singapore, Singapore
13th International Conference on Emergency Medicine

June 14
Kosice, Slovakia
Gastro-intestinal Models in the Research of Probiotics and Prebiotics-Scientific Symposium

June 16-19
Hong Kong, China
ILTS: International Liver Transplantation Society ILTS Annual International Congress

June 20-23
Mannheim, Germany
16th World Congress for Bronchoesophagology-WCBE

June 25-29
Orlando, FL, United States
70th ADA Diabetes Scientific Sessions

August 28-31
Boston, Massachusetts, United States
10th OESO World Congress on Diseases of the Oesophagus 2010

September 10-12
Montreal, Canada
International Liver Association's Fourth Annual Conference

September 11-12
La Jolla, CA, United States
New Advances in Inflammatory Bowel Disease

September 12-15
Boston, MA, United States
ICAAC: Interscience Conference on Antimicrobial Agents and Chemotherapy Annual Meeting

September 16-18
Prague, Czech Republic
Prague Hepatology Meeting 2010

September 23-26
Prague, Czech Republic
The 1st World Congress on Controversies in Gastroenterology & Liver Diseases

October 07-09
Belgrade, Serbia
The 7th Biannual International Symposium of Society of Coloproctology

October 15-20
San Antonio, TX, United States
ACG 2010: American College of Gastroenterology Annual Scienitfic Meeting

October 23-27
Barcelona, Spain
18th United European Gastroenterology Week

October 29-November 02
Boston, Massachusetts, United States
The Liver Meeting® 2010--AASLD's 61st Annual Meeting

November 13-14
San Francisco, CA, United States
Case-Based Approach to the Management of Inflammatory Bowel Disease

December 02-04
San Francisco, CA, United States
The Medical Management of HIV/AIDS

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5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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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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EDITORIAL

Safety of the long-term use of proton pump inhibitors

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Abstract

The proton pump inhibitors (PPIs) as a class are remarkably safe and effective for persons with peptic ulcer disorders. Serious adverse events are extremely rare for PPIs, with case reports of interstitial nephritis with omeprazole, hepatitis with omeprazole and lansoprazole, and disputed visual disturbances with pantoprazole and omeprazole. PPI use is associated with the development of fundic gland polyps (FGP); stopping PPIs is associated with regression of FGP. In the absence of *Helicobacter pylori* infection, the long-term use of PPIs has not been convincingly proven to cause or be associated with the progression of pre-existing chronic gastritis or gastric atrophy or intestinal metaplasia. Mild/modest hypergastrinemia is a physiological response to the reduction in gastric acid secretion due to any cause. The long-term use of PPIs has not been convincingly proven to cause enterochromaffin-like cell hyperplasia or carcinoid tumors. PPIs increase the risk of community acquired pneumonia, but not of hospital acquired (nosocomial) pneumonia. There is no data to support particular care in prescribing PPI therapy due to concerns about risk of hip fracture with the long-term use of PPIs. Long-term use of PPIs does not lead to vitamin B12 deficiencies, except possibly in the elderly, or in persons with Zollinger-Ellison Syndrome who are on high doses of PPI for prolonged periods of time. There is no convincingly proven data that PPIs increase the risk of *Clostridium difficile*-associated diarrhea in persons in the community. The discontinuation of PPIs may result in rebound symptoms requiring further and even continuous PPI use for suppression of symptoms. As with all medications, the key is to use PPIs only when clearly indicated, and to reassess continued use so that long-term therapy is used judiciously. Thus, in summary, the PPIs are a safe class of medications to use long-term in persons in whom there is a clear need for the maintenance of extensive acid inhibition.

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Key words: Acid inhibition; Drug safety; Osteoporosis; Pneumonia; Enteric infections

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INTRODUCTION

The risk of minor adverse effects from proton pump inhibitors (PPIs) is low, approximately 1%-3%, with rates of withdrawal from clinical research studies being 1%-2%, with no significant differences noted between the PPIs[1-6]. The risk of symptomatic adverse effects with the PPIs is low as well. In pooled data from published trials involving 2812 patients, omeprazole was

reported as causing headache (2.4%), diarrhea (1.9%), nausea (0.9%), and rash (1.1%), a profile similar to that of cimetidine and ranitidine[7]. In a prospective follow-up study of 5669 patients on lansoprazole, the most common reported adverse effects were diarrhea (4.1%), headache (2.9%), and nausea (2.6%). A similar profile has been reported for pantoprazole: diarrhea, 1.5%; headache, 1.3%; dizziness, 0.7%; pruritus, 0.5%[8]; rash, 0.4%[9]; and nausea, 0.015%. Compiled data from 3556 patients taking rabeprazole for up to one year demonstrated that the most common adverse effect was headache with an incidence (2.4%) similar to placebo (3.1%).

Serious adverse events are rare, with case reports of interstitial nephritis with omeprazole, hepatitis with omeprazole and lansoprazole[10-12], and disputed visual disturbances with pantoprazole and omeprazole[13,14]. An anticipated physiological effect of acid suppression with PPIs is an elevated serum gastrin concentration, which occurs with all PPIs. Gastrin elevation may be higher with omeprazole than with pantoprazole, and higher with lansoprazole than with omeprazole[15-17], and higher with rabeprazole than with omeprazole[18]. This variation in PPI-associated elevation of gastrin concentration is not clinically relevant.

Some persons with dyspeptic conditions such as gastroesophageal reflux disease (GERD) may need to be maintained long-term on PPIs. For this reason, we have reviewed the literature on possible long-term adverse effects of PPIs. Narrative or qualitative reviews, when compared to their systematic review cousins, trade off depth in favor of breadth. After all, reviews can be comprehensive and primary, or narrow in scope and heavily guarded against various biases. Narrative reviews remain, despite a heavy battering by hordes of high quality randomized controlled trials and mathematically endowed structured reviews, on their pedestal as a premier venue for medical educators and historians. This review fits in that important tradition, and purports to fill a need for a comprehensive review on the safety of long-term use PPIs. Mulrow published criteria for minimizing bias in narrative reviews[19]. Deeks summed up these as being rigorous, informative, comprehensive, and explicit[20]. Collins and Fauser, in their editorial, enforced the view of the importance of "balancing the strengths of systemic and narrative reviews"[21]. To achieve this balance and complement a primarily journalistic approach, a search of PubMed, Google Scholar and UpToDate for articles published since 1999 on the topic of "PPI" and "safety" (and related MESH terms) was conducted to identify English language meta-analysis, publications in one of the top biomedical journals in this field (NEJM, Annals, Lancet, JAMA, American Journal of Gastroenterology, Gastroenterology, American Journal of Gastroenterology, Alimentary Pharmacology and therapeutics, Drugs, BMJ) as well as major North American and European guidelines. Checklists have been proposed for systematic and qualitative research[22,23], aiding in the review. We present this information on the long-term safety of PPIs with a series of questions, a summary of the literature, and our proposed answer.

THE USE OF PPIS IS NOT ASSOCIATED WITH AN ALTERATION OF GASTRIC HISTOLOGY

In *Helicobacter pylori* (*H. pylori*) negative persons PPIs do not worsen pre-existing gastritis[24,25], and may even improve pre-existing gastritis[26]. PPIs do not cause atrophic gastritis[27,28].

In contrast, in *H. pylori* positive persons, *H. pylori* is associated with antral or body acute or chronic gastritis, atrophy and metaplasia[29]. *H. pylori*-associated chronic gastritis may progress to gastric atrophy, intestinal metaplasia, and gastric cancer[25,27,30,31], or may not[32]. *H. pylori* and PPIs may cause progression or acceleration from gastric antrum-predominant chronic gastritis to body-predominant chronic gastritis, and it is controversial whether gastric body-predominant atrophic gastritis (gastric atrophy) is a risk factor for gastric cancer[33]. *H. pylori* eradication may cause regression of gastric atrophy or intestinal metaplasia[26,27,29,34-37] or may not[33,38-44].

Thus, the long-term use of PPIs has not been convincingly proven to cause or accelerate the progression of pre-existing chronic gastritis, corpus gastric atrophy or intestinal metaplasia.

PHYSIOLOGICAL HYPERGASTRINEMIA DOES NOT CAUSE GASTRIC CARCINOIDS OR CANCER

H. pylori infection (without use of PPIs) itself increases serum gastrin concentration[29,45].

PPIs modestly increase serum gastrin concentration in persons who are *H. pylori*-negative or positive[46,47]. While PPI's may increase apoptosis[48], PPIs do not increase risk of gastric or esophageal cancer[49-51].

It is controversial whether the hypergastrinemia associated with the use of PPIs increase enterochromaffin-like (ECL) cell numbers, as well as linear or micronodular hyperplasia - Yes[52-54] in *H. pylori* positive persons[55], and No[43,56,57]. Hypergastrinemia associated with Zollinger-Ellison Syndrome (ZES), rarely is associated with an increase in ECL cell growth or ECL carcinoid[58]. Furthermore, there is only one published report in world literature of a ZES patient treated with PPIs for associated gastric hypersecretion, who developed gastric cancer[59]. This is probably a chance association.

Thus, mild/modest hypergastrinemia is a physiological response to a reduction in acid secretion due to any cause. The long-term use of PPIs has not been convincingly proven to cause ECL cell hyperplasia or carcinoid tumors. Even when hypergastrinemia is marked and prolonged (such as with ZES or MEN-1), gastric carcinoids are rare.

THE USE OF PPIS IS ASSOCIATED WITH THE DEVELOPMENT OF FUNDIC GLAND POLYPS

PPI use is associated with parietal cell hyperplasia, and an

up to fourfold increased incidence of fundic gland polyps (FGP)[60-62]. FGP also occur in the presence of *H. pylori* infection, likely incidentally[63,64]. Eradication of *H. pylori* or stopping long-term use of PPIs is associated with regression of FGP[62,65,66]. FGP in sporadic cases is rarely associated with dysplasia, but never gastric adenocarcinoma[67]. Dysplasia may occur in 25%-44% of gastric polyps in persons with familial adenomatous polyposis[63,68].

In summary, PPI use is associated with the development of FGP. FGP occur in the presence or absence of *H. pylori* infection. The eradication of *H. pylori* or stopping PPI is associated with regression of FGP. FGP may rarely become dysplastic, but almost exclusively this rare event is seen in persons with familial adenomatous polyposis.

PPIs may mask the symptoms of gastric cancer (GC), heal malignant gastric ulcers, or shorten survival in the patient with GC.

PPIs may mask the symptoms or heal early GC, but there is no data on the effect of PPIs on rates of survival[69]. H2RA's may[70] or may not[71] actually produce longer survival in patients with GC.

BIOAVAILABILITY OR METABOLISM OF A FEW OTHER DRUGS

PPIs reduce gastric acid, and thereby reduce the bioavailability of drugs requiring intragastric acidity to maximize their absorption and bioavailability[51]. Examples of such drugs would include ketoconazole, itraconazole and indinipur[72], and may reduce the effects of locally acting drugs such as sucralfate.

PPIs may alter the intestinal first pass metabolism or the hepatic clearance of some drugs, and thereby modify their pharmacodynamics[72]. They have no effect on n-acetyltransfer or xanthine oxidase activities[73], and may show a rare class action effect on vitamin K antagonists[74]. PPIs have a low drug interaction through phase I / II effects[5,75], and may differ in their possibility of causing drug interactions. Omeprazole and lansoprazole have a high affinity for CYP2C19 and CYP3A4 but these cytochromes contribute little to rabeprazole metabolism. Pantoprazole is completely metabolized by these cytochrome enzymes, but it uniquely has no drug interactions with a wide range of drugs[72,76-78].

PPIs, with the exception of pantoprazole, have been associated with reduced effectiveness of clopidogrel and a resulting 40% increased risk of coronary stent occlusions[79]. There is no consensus yet on how to manage this[80].

Thus, PPIs have an effect in common with all acid lowering therapy to reduce the absorption of acid-dependent medications. The metabolism of PPIs by hepatic cytochrome enzymes varies significantly between drugs.

THE USE OF PPIS AND DEFICIENCIES IN IRON AND VITAMIN B12

Iron

PPIs reduce gastric acidity, and in patients treated long-term with high dose PPIs duodenal absorption of organic and non-organic iron may be reduced[81]. This effect however is small, and PPIs are not associated with an increased risk of latent iron deficiency or iron deficiency[82].

Vitamin B12

PPIs reduce gastric acidity, which is necessary to activate pepsinogen to pepsin to release vitamin B12 from B12-containing foods. PPIs used short-term may minimally reduce the absorption of protein-bound B12 in food[83-85]. In elderly patients who may already have gastric atrophy (possibly from *H. pylori* infection), PPIs used long-term may reduce serum vitamin B12 concentrations[85-87]. Five out of six studies have shown that PPIs used long-term in non-elderly patients do not reduce serum vitamin B12 concentrations, and therefore body B12 stores[81,88-93].

In ZES patients treated long-term with high dose PPIs, the serum concentration of vitamin B12 may be reduced[94]. And yet, in cystic fibrosis (CF) children with reduced secretion of pancreatic bicarbonate and increased duodenal acidity, there is no reduction in the intestinal absorption of B12[76].

Thus, long-term use of PPIs does not lead to vitamin B12 deficiencies, except possibly in the elderly or in persons with ZES who are on high doses of PPI for prolonged periods of time[95,96].

RISK OF OSTOPENIA, OSTEOPOROSIS AND WITH BONE METABOLISM

PPIs alter osteoclastic vacuolar mechanisms which may reduce bone absorption[97], and thereby actually reduce the risk of OP. PPIs have no known adverse effect on vitamin D absorption or metabolism.

What is the link between PPI use and metabolic bone disease? There is a highly variable effect of acid suppression on calcium absorption[98,99]. In persons with achlorhydria due to pernicious anemia, Ca^{2+} absorption is normal or reduced[100,101].

The real question is whether PPI use is associated with an increased risk of osteoporosis/osteopenia, and more importantly with bone fractures. In case controlled studies, PPI use long-term is associated with an increased risk of bone fractures, and this increased risk depends on the duration and dose of chronic use of the PPI[102] (e.g. Manitoba Population Health Research Data Repository[103]). Use of PPI $\geqslant$ 5 years can increase the risk of osteoporotic fractures by 1.62-fold (95% CI: 1.02-2.58). Other studies confirm that use of PPI $\geqslant$ 7 years increases the risk of osteoporotic hip fractures by 4.55-fold (95% CI: 1.68-12.29) and PPI use for 6-12 mo has been reported to be associated with an increased risk of osteoporotic hip and spine fractures[103-105]. Osteoporotic fractures of the hip and spine may be associated with many factors, which must be carefully taken into account in any case-controlled study which suggests a new association, such as the use of PPIs. However, case control studies on the risk of OP may be criticized on a methodological basis, such as the lack of appropriate stratification of the risk of other

factors known to be associated with an increased risk of OP[106]. The Canadian Association of Gastroenterology (CAG) position paper suggests that "current data would not support particular care in prescribing PPI therapy due to concern about risk of hip fracture"[107].

COMMUNITY ACQUIRED NOSOCOMIAL PNEUMONIA

What is the physiological background to speculating that PPIs might result in pulmonary complications? PPI use is associated with increased intragastric aerobic bacteria, and with the production of acetaldehyde from alcohol[108]. The increased bacterial colonization of the stomach observed with PPI users may be associated with pulmonary micro-aspiration and lung colonization[109,110]. In addition, it is postulated that secretions from the oropharynx may pass by micro-aspiration into the lower lung airways[111]. Furthermore, lung colonization may occur as a result of mechanisms other than micro-aspiration of gastric contents, because different organisms may grow from cultures of gastric juice and from bronchoalveolar lavage[112].

From the clinical perspective, the risk of community acquired pneumonia (CAP) is 0.6 per 100 person years. In persons on PPIs, the odds ratio (OR) is 1.89 (95% CI: 1.36-2.62) for current PPI use and 1.5 (95% CI: 1.3-1.7) for past PPI use (95% CI: 0.9-1.6, and 0.8-1.3, respectively)[113].

In the short-term, PPI use increases the risk of CAP: use of PPI for 2 d, OR = 6.53 (95% CI: 3.95-10.80); for 7 d, OR = 3.79 (95% CI: 2.65-5.42); for 14 d, OR = 3.21 (95% CI: 2.46-4.18); but long-term use of PPIs does not increase the risk of CAP[114], and furthermore meta-analyses have shown that there is no significant association between PPIs and CAP[115].

In contrast, PPIs do not increase the risk of hospital acquired (nosocomial) pneumonia (NP). In fact, there is a reduced risk of NP in patients with nasogastric tubes on a PPI[116]. For ventilated pediatric patients in ICU, there is no increased risk of NP[117-119].

Thus, short-term PPI use increases the risk of CAP, but PPI use does not increase the risk of hospital acquired pneumonia.

CLOSTRIDIUM DIFFICILE-ASSOCIATED PNEUMONIA

There are numerous risk factors for CDAD (use of antibiotics, age, contact with an infected patient or healthcare worker, crowding, lack of cleanliness, post-pyloric tube feeding, patient immunosuppression)[120]. These factors must be taken into account for the attribution of risk, e.g. before assigning a possible role to a new factor, such as PPIs. Some observational studies show an association between PPI use and risk of CDAD[121-132]. For example, for PPI use and CDAD in chronic renal failure patients, the AOR is 5.7 (95% CI: 1.3-39.1) (P = 0.02)[133]. In meta-analyses of studies of CDAD and PPIs, the AOR is 1.96 (95% CI: 1.28-3.00). Some of these reports involve a hypervirulent strain of *C. difficile*, and after correcting for other factors such as antibiotic use, there is no association with PPIs[134]. The bottom line is that there is no convincingly proven data that PPIs increase the risk of CDAD[134,135].

SMALL BOWEL CONTAMINATION SYNDROME AND ENTERIC INFECTIONS

It is thought that PPIs have a minor effect on altering the intestinal bacterial microbiota[136]. Observational studies have suggested that PPIs may[137] or may not[138] increase risk of enteric infections.

Thus, PPIs do not have a convincingly proven adverse effect on the enteric microbiota, and if such an effect does exist, there is no proven clinically important adverse effect[139,140].

The use and subsequent withdrawal of PPIs may be associated with an exaggeration of, or new onset of, acid-related symptoms. PPIs are a medication that is generously prescribed for a variety of symptoms that are thought, and not necessarily confirmed, to be acid-induced. One reason for this is the relatively low number of adverse effects that have been shown in the short- or long-term. One study suggests that symptoms that commence following the discontinuation of PPIs due to rebound acid hypersecretion may be as troublesome as the symptoms that the PPIs were being used to treat in the first place[141]. Because of these rebound symptoms, there may be a need for further and continuous PPI use. As with all medications, the key is to use PPIs only when clearly indicated, and to reassess continued use so that long-term therapy is used judiciously.

The risk of false-negative urea breath tests (UBT) for the diagnosis of an *H. pylori* infection is lower for pantoprazole[142,143]. While it is recommended that acid suppression therapy should be discontinued prior to a UBT, the false-negative effect is lower for pantoprazole.

The biological plausibility is poor for the possibility that PPI use is associated with an increased risk of colorectal cancer or adenomatous polyps, and there is no clinical data to suggest this possibility.

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EDITORIAL

Double balloon enteroscopy and acute pancreatitis

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Abstract

Double balloon enteroscopy (DBE) is a new technique, first published and introduced into clinical practice in 2001 by Yamamoto, the inventor of this outstanding method. DBE allows complete visualization, biopsy and treatment of the small bowel. Nowadays, we have some experience of this method for evaluation of the complication rate. Severe complications are described in 1%-1.7% of patients. Acute pancreatitis is a rare complication of the investigation. The incidence of acute pancreatitis after diagnostic DBE is 0.3% in most studies. More than 50 cases of acute pancreatitis have been described in the literature so far. On the contrary, hyperamylasemia after DBE seems to be a rather common condition. Association with acute pancreatitis is supposed to be possible, but not obligatory. The causal mechanism of post-DBE acute pancreatitis is uncertain, and there are several theories in the literature. The most probable cause seems to be a mechanical straining of the endoscope with over-tube on the pancreas or in the papillary area.

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Key words: Double balloon endoscopy; Gastrointestinal endoscopy; Small intestine; Hyperamylasemia; Acute pancreatitis

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INTRODUCTION

Double balloon endoscopy (DBE) is a method of enteroscopy that was introduced 8 years ago. Despite 8 years of experience, the complication rate is still under evaluation. Acute pancreatitis is the most feared complication in oral DBE. The cause of acute pancreatitis is uncertain. The aim of this paper is to provide an in-depth overview of possible risk factors for acute pancreatitis in DBE.

HISTORY

The small intestine was inaccessible to endoscopic methods for a long time. Too far from the mouth and the anus, it seemed to be unreachable for the endoscopist. The history of endoscopy investigation of the small bowel is quite short but accompanied by long-lasting skepticism.

In 1999, Mosse and Swain still stated in their work: "Enteroscopy remains the procedure in the gastrointestinal tract that is most inaccessible to endoscopy, and technical limitations severely impair the ability to advance and examine the small bowel reliably or completely"[1].

In 2000, Oates and Morris[2] published in their article: "It is now more than 25 years since small bowel enteroscopy was first described. For several reasons, this technique developed more slowly than other more usual forms of endoscopy. The small bowel disease is relatively rare in comparison with other gastrointestinal diseases. Also, there

was lack of initial design agreement in different types of enteroscopes. Finally, commercial interests of the manufacturers of endoscopes were mainly focused on the more conventional, large volume markets. Problem areas remain, but with advancing technology and more professional interest in this area, these will be addressed during the next few years"[2].

Attempts to observe the entire gastrointestinal tract began even with early fibroscopes, but only two applicable methods were developed in addition to intra-operative enteroscopy: the ropeway method described by Deyhl *et al*[3] and Classen *et al*[4] in 1972 and the sonde endoscope described by Tada *et al*[5] in 1977. The first successful total enteroscopy was performed in March 1971 by Hiratsuka, using the ropeway method[6]. Both methods were soon abandoned due to the complexity of the technique, patient discomfort, and the long time needed to complete the procedure (and the high rate of complications of ropeway enteroscopy).

Push enteroscopy using a long endoscope was regarded as the gold standard then, but most of the small intestine remained beyond its reach. The first procedure was performed by Parker using a colonoscope in 1983[7]. Push enteroscopy can definitely not evaluate the non-resected small bowel in its entire length. Nowadays, push enteroscopy is reserved exclusively for investigation of the duodenum and oral end of the jejunum[8].

Recent innovations and introduction of two new methods (wireless capsule endoscopy and DBE) have made observation of the entire small intestine possible[6]. Both of these techniques are now available in clinical practice and are complementary: capsule endoscopy for screening and DBE for further diagnostics and/or therapy.

Capsule enteroscopy was invented by Swain and initial experiences in 4000 patients were published by Fritscher-Ravens and Swain in 2002[9]. The introduction of DBE for investigation of the small bowel in 2001 was a milestone in gastrointestinal endoscopy because it allows us to carry out therapeutic interventions as well as diagnostic procedures in the small bowel[10,11].

Double balloon (push-and-pull) enteroscopy (Fujinon, Inc., Saitama, Japan) represents an endoscopic method that enables us to investigate a substantial part or even the entire small bowel. The device was developed by Yamamoto and colleagues, introduced by him into clinical practice, and their first experiences were published in 2001[12]. Several subsequent studies[13-18] have suggested that this method is feasible for diagnostic and therapeutic purposes. Nevertheless, DBE is still under evaluation and its yield and safety aspects must be further determined[19-22].

Single balloon enteroscopy (SBE) is a modification of DBE, and is another system for small bowel enteroscopy (Olympus, Tokyo, Japan). The endoscope (XSIF-Q160Y) consists of a high-resolution enteroscope with a working length of 200 cm. The device is equipped with a transparent silicone overtube with a silicone balloon attached to its distal part. In contrast to the DBE device, there is no balloon attached to the enteroscope, and therefore stable position of the device must be maintained by hooking the distal tip of the enteroscope on the small-bowel wall[23].

Balloon-guided enteroscopy (BGE) is another modification of the DBE method. The advantage of this novel push-pull technique is that it is cheaper than other balloon-assisted methods (DBE and SBE). The device can be used with standard endoscopic equipment. The BGE device comprises a double balloon added onto a disposable element and an air supply unit (NavAid ASU; Smart Medical Systems, Ra'anana, Israel) to control the inflation and deflation of the balloons. The disposable BGE element is slipped over the tip of a standard endoscope and then fixed to the endoscope. At the tip of the endoscope is an inflatable stabilizing balloon. During diagnostic or therapeutic interventions through the endoscope's instrument channel, the advancing catheter with its front balloon deflated can be pulled back and "parked" in its dedicated channel in the BGE device[24].

Spiral enteroscopy is a new technique for deep small-bowel intubation that uses a special overtube (Endo-Ease Discovery SB; Spirus Medical, Stoughton, MA, USA) to pleat the small bowel. Any type of enteroscope can be passed through the overtube, which has helical spirals at its distal end and rotates independently from the endoscope. The enteroscope can be locked in the overtube, which allows the option of spiral enteroscopy, or unlocked and advanced through the overtube[25,26]. Now, it is necessary to gain more data on its usefulness and safety and to compare this method with balloon-assisted enteroscopy[27].

Intra-operative enteroscopy is still a useful method for a specific group of patients (in case of failure of DBE, adhesions, multiple small transmural lesions unresolvable by endoscopic methods, such as carcinoids, and blue rubber bleb nevus syndrome), and therefore, it is necessary to be able to use this method in carefully considered cases[28-31].

PARTICULARITY OF DBE

As DBE is a lengthy procedure, a large volume of air is usually insufflated, which leads to significant distension of the small bowel (with the formation of distended bowel loops and acute angulations with increasing amounts of gases intraluminally). CO_2, unlike air, is rapidly absorbed from the bowel. Preliminary data indicate that bowel insufflation with CO_2, instead of air, enhances patient comfort and decreases the need for sedation[23,31]. We have used CO_2 insufflation in DBE procedures regularly from 2007. We had no complications with hyperinflation, and the comfort of the patient rapidly increased. This type of insufflation is helpful for easier and deeper insertion of the endoscope, because the absorption of CO_2 is 150 times faster than absorption of air in the bowel. Indeed, a recent randomized double-blind trial showed that insufflation with CO_2 is safe, reduces patient discomfort, and significantly improves intubation depth[32].

Combination of water with simethicone is used rou-

tinely to do away with bubbles in the intestine. During withdrawal of the endoscope and during therapeutic interventions, spasmolytics might improve visualization of the small-bowel mucosa by reducing motility of the small bowel[23]. We administer intravenous crystalloids, mostly saline solution, to all patients during DBE lasting over 30 min. Conscious sedation is thought to be sufficient for DBE[23]. It seems to be much better in DBE in comparison with general anesthesia, according to our experience. Abdominal pain is an important warning signal, and it is necessary to terminate the procedure immediately in such cases. Intense pain may be a sign of inadequate pressure on the pancreas and poses a high risk of post-DBE pancreatitis[21,22]. We use small intravenous repetitive doses of midazolam and pentazocine for conscious sedation (batchwise). The duration of the procedure and discomfort for the patient caused by oral passage of the overtube requires deep analgosedation. The procedure requires an experienced endoscopist and the possibility of fluoroscopy if needed, especially during the learning period[33].

COMPLICATIONS

DBE has been reported as a safe endoscopic technique. In initial series on DBE, no complications during or after the procedure were reported[34-37]. Recently, the overall complication rate is stated as being about 1.7%[38,39].

A complication of endoscopy is defined as any event that negatively changes the health status of the patient, and that occurs during the 30-d period after the investigation. Complications are usually categorized as minor when requiring up to 3 d of hospitalization, moderate when requiring 3-10 d and major or severe when requiring > 10 d, and/or an endoscopic, radiological or surgical intervention, and/or contribute to the death of the patient[38,40]. Procedure-related mortality is defined as mortality within 30 d of DBE[38]. Complications are divided into two main categories, those directly attributed to the procedure and those secondary to anesthesia or conscious sedation[23]. The most common complications secondary to anesthesia or conscious sedation are respiratory depression, aspiration, and pneumonia, with a frequency of < 1%[23].

Until now, no standards or definitions for complications during or after DBE have been established. Potential complications during or after DBE might be: perforation, bleeding, balloon dislocation, sedation-related, segmental enteritis after argon plasma coagulation[41], intestinal necrosis after epinephrine injection[42], paralytic intestinal ileus[43], and acute parotitis[44].

Recently, post-DBE pancreatitis has been recognized as a complication[39,45]. In diagnostic procedures *via* the anterograde approach, pancreatitis is the most common and most severe complication[23]. The very first post-DBE acute pancreatitis was reported by Honda *et al*[46] in 2006.

An international symposium held in Atlanta, GA, USA in 1992 has established a clinically based classification system for acute pancreatitis[47,48]. The goal was to establish international standards for definition of acute pancreatitis and its complications, to facilitate valid comparisons of severity of illness and results of therapy, and also to establish criteria for patient selection in randomized prospective trials. According to the Atlanta symposium, acute pancreatitis is defined as an acute inflammatory process of the pancreas that may also involve peripancreatic tissues and/or remote organ systems. Mild acute pancreatitis is defined as pancreatitis associated with minimal organ dysfunction and uneventful recovery. Severe pancreatitis is defined as pancreatitis associated with organ failure and/or local complications (necrosis, abscess, or pseudocyst). Criteria for severity included organ failure (particularly shock, pulmonary insufficiency, and renal failure) and/or local complications (especially pancreatic necrosis but also including abscess and pseudocyst). Early predictors of severity within 48 h of initial hospitalization included Ranson signs and APACHE Ⅱ (Acute Physiology and Chronic Health Evaluation Ⅱ) points[47-49].

In the Atlanta symposium, a uniform threshold was not established for serum amylase and/or lipase for the diagnosis of acute pancreatitis. In recently published articles, the threshold varies from ⩾ 2 to ⩾ 4 times the upper limit of normal. Criteria for severe pancreatitis include organ failure and/or local complications. This broad definition describes a heterogeneous group of patients with varying levels of severity. For example, the prognosis of pancreatic necrosis is more serious than a pseudocyst or pancreatic abscess. Also, almost all patients with necrotizing pancreatitis without organ failure survive, whereas those with multisystem organ failure do not[49].

Bollen *et al*[50] have revised the Atlanta symposium in their review. The authors propose the following recommendations for revision of the classification of acute pancreatitis. (1) The diagnosis should incorporate two of the following three items: upper abdominal pain, amylase and/or lipase levels ⩾ 3 times the upper limit of normal (as this cut-off is used most frequently in the literature), and computed tomography (CT) or magnetic resonance imaging findings compatible with acute pancreatitis; (2) Persistent organ failure (for at least 48 h) should have an important role in defining the severity of acute pancreatitis; and (3) Decisions should be made as to which predictive scoring system, including cut-off value, should be used to define predicted severe acute pancreatitis, based on a systematic review of the available data.

Progress in the field of acute pancreatitis is hampered greatly when various author groups use their own idiosyncratic definitions[50].

According to the literature on post-endoscopic retrograde cholangiopancreatography (ERCP) pancreatitis [American Society for Gastrointestinal Endoscopy (ASGE) guidelines], post-DBE pancreatitis is defined as newly developed or worsened abdominal pain after the procedure, with a serum amylase ⩾ 3 times the upper limit of normal as the upper limit 24 h after the procedure and requiring at least 2 d of unplanned hospitalization after the procedure[51]. According to these guidelines, the severity of the disease has been classified as follows: mild, requiring 2-3 d

hospitalization; moderate, 4-10 d hospitalization; and severe, > 10 d hospitalization, and/or the occurrence of pseudocyst and/or the need for surgery[52]. The duration of pain after the procedure is crucial for defining post-endoscopy pancreatitis[53].

We found the definition from UpToDate 2009 to be fundamental. Acute pancreatitis is an acute inflammatory process of the pancreas. It is usually associated with severe acute upper abdominal pain and elevated blood levels of pancreatic enzymes. Acute pancreatitis can be suspected clinically, but requires biochemical, radiologic, and sometimes histological evidence to confirm the diagnosis. Clinical, biochemical, and radiologic features need to be considered together since none of them alone is diagnostic of acute pancreatitis. Acute pancreatitis is an important cause of acute upper abdominal pain. Because its clinical features are similar to a number of other acute illnesses, it is difficult to base a diagnosis only on symptoms and signs. The disease varies in severity and the diagnosis is often missed at the extreme ends of the spectrum[54].

It is not usually difficult to recognize severe pancreatitis. The mild form may pose a problem. Abdominal pain and hyperamylasemia after DBE need not mean pancreatitis. On the other hand, a lot of mild pancreatitis could be missed in patients with DBE performed on an outpatient basis.

The complication rate of diagnostic procedures is low (0.4%-0.8%) according to the literature[10,11,38,45]. The overall complication rate of therapeutic DBE is 3%-4%. However, difficult therapeutic endoscopic procedures (e.g. resection of large polyps) may increase the risk to 10%[10,11,23,38]. The perforation rate is significantly elevated in patients with postsurgical anatomical changes undergoing diagnostic retrograde DBE examinations[55].

The overall complication rate was reported to be about 1.7% in a recent international multicenter survey of 2362 DBE procedures. The complications were rated minor in 0.9%, moderate in 0.3% and severe in 0.6% of procedures. The complication rate is significantly higher in therapeutic procedures in comparison with diagnostic ones (4.3% *vs* 0.8%). An exception to the rule is acute pancreatitis, the most common complication in diagnostic DBE procedures. Acute pancreatitis was reported in 0.3% of DBEs[38,39].

A recent report from the National German DBE Register showed an overall complication rate of 1.2% in a large series of 3894 DBE procedures. The incidence of acute pancreatitis was also 0.3% in that study[11].

A study by May *et al*[41] evaluated the acute complication rate of DBE in 353 patients. Only therapeutic procedures were evaluated, with a complication rate of 3.4%. No acute pancreatitis was reported[41].

In general, DBE is associated with a higher complication rate compared with standard endoscopic procedures.

OUR OWN EXPERIENCE

We began with DBE in our department in March 2006. All of our investigations were performed using EN-450T5 and EN-450P5 endoscopes (Fujinon, Inc., Saitama, Japan). Being aware of the possibility of acute pancreatitis as a complication of DBE, we started with our prospective study on hyperamylasemia after DBE right from the start of DBE in 2006. This prospective study is still continuing. All patients were admitted to our department and followed prospectively for at least 2 d after oral DBE.

Urine and serum amylase, serum pancreatic lipase and C-reactive protein (CRP) were investigated before and 4 h and 24 h after DBE. Abdominal pain was evaluated using a three-step scale. Hyperamylasemia (exceeding the upper normal limit) and marked hyperamylasemia (reaching ⩾ 3 times the upper limit of normal) were distinguished.

Normal ranges of the following values in our laboratory were: serum amylase 0.44-1.67 μkat/L (i.e. 28-100 U/L); urine amylase 0-7.67 μkat/L (i.e. 0-460 U/L); serum lipase 0.22-1 μkat/L (i.e. 13-60 U/L); and serum CRP 0-5 mg/L.

Acute pancreatitis was diagnosed in accordance with clinical signs (abdominal pain, fluid sequestration and lack of peristalsis), CRP level and CT scan in addition to the above.

Risk factors for acute pancreatitis and the importance of hyperamylasemia associated with DBE have not been satisfactorily resolved yet. The aim of our prospective study was to clarify the relationship between oral DBE and amylasemia and lipasemia, and to address the possible role of the learning curve for DBE for risk of acute pancreatitis.

A total of 138 DBEs were carried out from March 2006 to November 2009 in 60 men and 56 women under deep conscious sedation (midazolam and pentazocine). The mean time of DBE was 110 min (range 12-270 min), and the mean number of push-and-pull cycles was 15 (range 1-47).

Amylase was set after 128 DBEs; elevation was found in 61 (48%), and marked hyperamylasemia in 27/128 (21%). Abdominal pain was recorded in 19/96 (20%). Elevated lipase levels were found in 55/94 (59%); including 38/94 (40%) with ⩾ 3 times the upper limit of normal. We observed elevation of CRP after DBE in only 18/100 (18%). Peak values of serum amylase and lipase levels were found 4 h after DBE, and peak values of CRP at 24 h after the procedure.

Total panenteroscopy (i.e. investigation of the entire small intestine by one oral approach in one session) was accomplished in 12 DBEs (9%). In this subgroup, we found only four patients with hyperamylasemia and two with marked hyperamylasemia. We had no complications in this subgroup. Compared to the total number of patients, in this subgroup, there was a younger mean age and longer duration of DBE (mean 148 min *vs* 106 min; $P = 0.010$), but there was no acute pancreatitis and no significant difference in amylase and lipase and/or abdominal pain.

In all DBEs, we did not identify any risk factor for abdominal pain and/or elevated pancreatic amylases (sex, age, previous abdominal surgery, panenteroscopy, indica-

tion or endoscopic finding, type of endoscope, number of push-and-pull procedures, diagnostic or therapeutic procedure).

Three patients (2.1%) developed acute pancreatitis after DBE, one necrotizing and two edematous. Two had inflammatory affection of the pancreatic tail, the third of the head region. All of them had abdominal tenderness during the procedure.

Subsequently, we divided our patients into two groups (the first 69 and second 69 procedures) to assess possible influence of the learning curve. Diagnostic and therapeutic DBEs (using Fujinon EN 450P5 or 450T5) were proportionally included in both groups. Neither group differed in age, sex, diagnoses or previous surgery. Differences in amylase and lipase (4 or 24 h after minus basal values before DBE) were used as a major indicator.

The difference in abdominal pain, DBE duration, amylase or lipase was not significant in either of the two groups. Marked hyperamylasemia (≥ 3 times above the upper limit; in 21% after DBE) was not associated with marked pain. There was a weak but significant correlation between amylase difference and abdominal pain (P = 0.003), the number of push-and-pull cycles (P = 0.018), and negative correlation with age (P = 0.029). Lipase difference 4 h after DBE weakly correlated with abdominal pain (P = 0.034). Our three cases of acute pancreatitis were numbers 24, 50 and 57 of 138 DBE procedures, without any evidence of further consequences. DBE lasted 12-270 min (mean: 110 min). Shorter (≤ 120 min, 64%) and longer (> 120 min; 50/138, 36%) DBEs did not differ in terms of post-DBE abdominal pain (P = 0.784). There was a borderline significant difference between longer and shorter DBEs for amylase (P = 0.047) but not lipase (P = 0.225) differences 4 h after DBE.

Elevation of amylase and lipase is often associated with DBE, but acute pancreatitis is however a rare complication. Duration of DBE is not a risk factor for post-DBE acute pancreatitis. Abdominal pain during DBE should be considered as a possible risk factor for acute pancreatitis. That is why we prefer conscious sedation to general anesthesia in oral DBE. The initial learning curve for DBE is not associated with higher amylase or lipase in our setting, and it does not signify a risk factor for post-DBE acute pancreatitis[21,22,56]. The most important point of our study was that it was prospective, solely on an inpatient basis, and all consecutive patients who underwent oral DBE were included.

CLINICAL OUTCOMES

Our center has long-term experience (since 1994) with push-enteroscopy[57] and intraoperative enteroscopy[28-31]. We have never registered acute pancreatitis as a complication of either push-enteroscopy or intraoperative enteroscopy in our setting. However, acute pancreatitis as a complication of push-enteroscopy, caused by an overtube, has been described previously by other authors[58]. Acute pancreatitis has even been described after uneventful upper and lower gastrointestinal endoscopy[59-61]. Blackwood *et al*[62] have detected asymptomatic hyperamylasuria in 6.6% of patients undergoing gastrointestinal endoscopy[62].

Pelletier *et al*[63] have studied the prevalence of hyperamylasemia 2 and 24 h after upper gastrointestinal endoscopy in 50 consecutive patients. In the 2-h sample, hyperamylasemia was observed in nine patients (18%), and in the 24-h sample, in five patients. Pelletier *et al*[63] have concluded that the cause of hyperamylasemia may be due to hypersalivation during the procedure. In our opinion, hypersalivation cannot affect the serum amylase level in such a way (most of the saliva runs out of the mouth during endoscopy and is not swallowed). Furthermore, it cannot affect abdominal pain or pancreatic lipase elevation[21,22,56].

DBE was initially described as a safe procedure[6], with the rate of severe complications ranging from 0 to 1.4%[14,18,64]. However, abdominal pain lasting 1-2 d occurred in 9% of patients in one study[13], or even in 20% according to another[14]. Abdominal discomfort reducing within 72 h was reported in three out of six patients after a DBE procedure[65].

There have been 51 published cases of post-DBE acute pancreatitis on PubMed to date.

Eisen and Schreiner[45] have presented a study of 275 consecutive patients who underwent DBE at two tertiary referral hospitals. The most common complication of DBE was abdominal pain which was seen in 20% of cases. This was typically self-limiting, yet a systematic analysis was not performed. Three cases of pancreatitis occurred (1%), two of which were mild and one of which was of intermediate severity[45]. It is hard to say how many patients with self-limited abdominal post-DBE pain had mild acute pancreatitis, because of inadequate follow-up.

Honda *et al*[46,66] described one and Groenen *et al*[67] two (one necrotizing) cases of acute pancreatitis after DBE. Heine *et al*[14] and Jarbandhan *et al*[52] from the same Dutch group have studied 603 DBE procedures (441 oral DBEs) on an outpatient basis, with six cases of post-DBE acute pancreatitis (1% of all DBEs, i.e. 1.4% of oral DBEs); all cases of pancreatitis were diagnosed after oral procedures. None of the cases of pancreatitis were in the head of the pancreas[52]. In a retrospective analysis of 378 DBEs by Zhong *et al*[68], two patients (0.7%) suffered from abdominal pain with an unspecified elevation of serum amylase. Unfortunately, it was not stated if they required hospitalization[68]. Möschler *et al*[10,11], in a German multicenter retrospective study, have reported 3894 DBEs with an overall complication rate of 1.2%. They quoted nine cases of acute pancreatitis with one lethal disease course after oral DBE, with a complication rate of 0.34%[10,11]. A retrospective study by Gerson *et al*[55] in nine United States centers collected data from 2478 DBEs, with a total of 0.9% major complications (22 DBEs), among which there were six cases of acute pancreatitis (0.2%). Surprisingly, one case of acute pancreatitis was reported after an anal DBE procedure[55]. Another multicenter study by Mensink *et al*[38] investigated 2362 DBEs. The majority of these (87%) were performed on an inpatient basis. The overall complication rate was 1.7%;

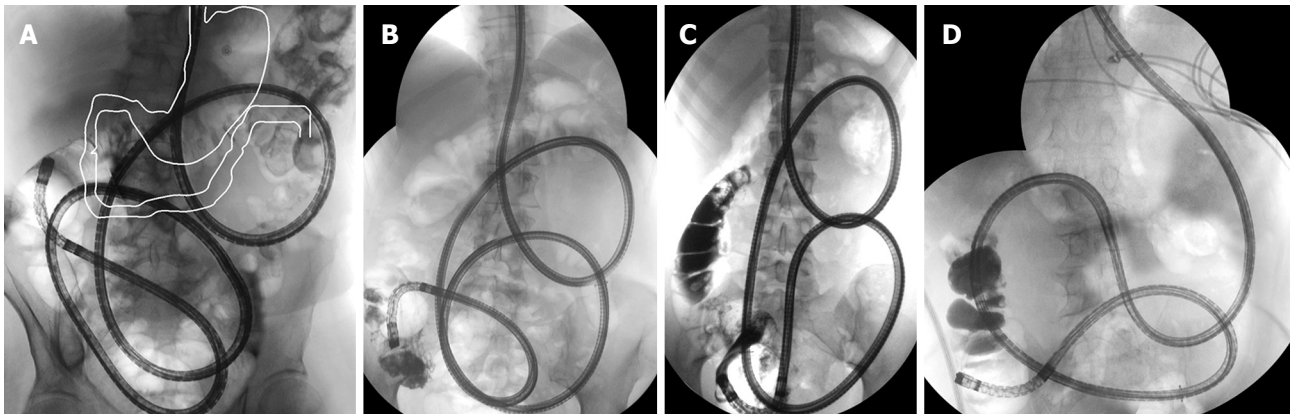


Figure 1 DBE, panenteroscopy *via* oral approach (plain radiograph) in supine position. In comparison with the normal situation (A, white outline), the duodenum is shifted markedly to the left, straightened and shortened. A-C: Normal anatomy; D: After B Ⅱ resection.

six patients suffered from acute pancreatitis (0.3%). The location of the pancreatitis was the body and/or the tail of the pancreas in four patients, the entire pancreas in two, and the head of the pancreas in one[38]. One case of post-DBE acute pancreatitis that affected the pancreatic tail has been reported at the Mayo Clinic (Rochester, MN, USA)[69], along with another case from Osaka, Japan[70], and Rotterdam (one case of pancreatitis of unknown location from 135 patients, 0.7%)[71]. In a recent study by Sunada and Yamamoto, four cases of acute pancreatitis in 1092 DBEs (0.37%) were diagnosed, although the severity and location were not mentioned[72].

In agreement with the Dutch study, we believe that post-DBE pancreatitis is underestimated in retrospective studies on an outpatient basis. In particular, retrospective questionnaire-based surveys might be at risk from an inaccurate report or inclusion bias[52]. As the distinction between clinically mild pancreatitis and hyperamylasemia with transient abdominal discomfort is somewhat arbitrary, it seems likely that an under-diagnosis of post-DBE pancreatitis might have occurred, especially in outpatients.

There was no association with sex, duration of the procedure, or type of endoscope in most of the studies mentioned above.

Surprising results have been reported by Pata *et al*[73] in their study of 48 oral DBE procedures. Pancreatitis was observed in six patients (12.5%). Acute pancreatitis was diagnosed when amylase and/or lipase reached ⩾ 3 the upper limit of normal in the presence of pancreatic-type abdominal pain[73]. The question is whether the diagnostic criteria of acute pancreatitis are sufficient in the case of post-DBE pancreatitis. Asymptomatic hyperamylasemia may occur in nearly half of DBE procedures[21,22,27,56,66,74]. Honda *et al*[66] have investigated 13 patients who underwent DBE. Hyperamylasemia occurred in six of them after the procedure, and one of these developed acute pancreatitis. In agreement with our results, this study demonstrates that latent hyperamylasemia without the development of pancreatitis occurs after peroral DBE more frequently than was previously thought[66]. It may be that some other criteria for the diagnosis of post-DBE acute pancreatitis should be added: ultrasound and/or CT scanning, peristalsis weakening, or compartment sequestration syndrome. Time will tell if the significance of post-DBE and post-ERCP enzyme elevation is absolutely comparable. Pata *et al*[73] have verified only two reported cases of pancreatitis using CT.

The causal mechanism of post-DBE acute pancreatitis is uncertain, and there are several hypotheses. Nevins assumes local trauma to the pancreas during the procedure or release of as-yet-undefined inflammatory mediators[59]. May *et al*[75] assume that the length of the examination time is an important factor, because they did not observe pancreatitis in more than 500 DBEs and had a strict maximum examination time limit of 2 h[75]. However, data from other authors (including our own data) do not support this assumption. In our study, in agreement with other studies[66,70], we found hyperamylasemia and pancreatitis in patients with shorter procedures, and we had fewer patients with hyperamylasemia and no case of pancreatitis in the subgroup of 12 patients with panenteroscopy *via* an oral approach (mean time: 148 min)[21,22,56]. We therefore believe, like Honda *et al*[76], that it is the technique of DBE itself, with the shortening of the small bowel, that may be a factor in pancreatitis after peroral DBE, rather than the length of the examination time (Figure 1)[21,22,56].

Honda *et al*[66] think that, in peroral DBE, the duodenum and proximal jejunum are markedly shortened, and the duodenum is sometimes found to be nearly straight from the pyloric ring to the ligament of Treitz on fluoroscopy. In these conditions, the pancreas body and/or tail may be subject to severe strain, with traumatic injury and/or ischemia. Intraluminal pressure in the duodenum may increase in such a way as to disturb the secretion of pancreatic juice. This mechanism might be associated with the occurrence of hyperamylasemia and pancreatitis after peroral DBE[66]. This hypothesis is supported by the finding that, in most cases in which early CT was performed, pancreatitis was located in the tail or body-tail region of the pancreas[52].

Heine *et al*[14] state that pancreatitis probably results from prolonged mechanical stress on the organ due to re-

peated stretching by the endoscope[14]. Sunada and Yamamoto suggest that the possible mechanism is mechanical torsion of the pancreatic body during insertion of the endoscope. Therefore, for oral insertion, extreme shortening should not be performed, and counter-clockwise rotation is preferred[72].

Another possibility is ischemia of the pancreas, which could induce acute pancreatitis. This has been confirmed in an animal model[77].

Groenen *et al*[67] hypothesize that acute pancreatitis is caused by an increase in intraluminal duodenal pressure during the endoscopic procedure caused by inflation of the two balloons, which leads to reflux of duodenal fluids into the pancreatic duct[67]. A closed duodenal loop has indeed become an established animal model for acute pancreatitis[78,79]. However, the question is whether the duodenal intraluminal pressure during DBE really can reach such a high level. On the other hand, the pancreatitis caused by reflux should affect the whole pancreas, with diffuse swelling, not only in the body or pancreatic tail.

Some endoscopists prefer to inflate the balloons after passing the ligament of Treitz[52,71], or after two insertions (being at least 50 cm distal from the papilla of Vater)[39]. Unfortunately, precise description of this technique and its control (by fluoroscopy?) was not incorporated in their work. Frankly, it is difficult to believe that by pushing the endoscope with the overtube, without pulling back and straightening out the looping, they were able to pass the ligament with a 129-cm long device (the balloon on the tip is 7 cm long and the rest of working part of the overtube is 129 cm). On the other hand, the papilla is about 80-90 cm from the incisors when inserting the endoscope, therefore, both balloons are probably beyond the papilla of Vater. However, even with this safe technique of endoscope insertion, the Dutch group had a post-DBE acute pancreatitis rate of 1.4%, using the oral approach[52].

Other possible causes are direct trauma to the ampullary area or a direct obstruction of the pancreatic duct by the insufflated balloon[67]. In our opinion, this hypothesis seems also to be unlikely.

Another consideration is the question of whether the elevation of amylase is always a result of pancreatitis or whether it could be of intestinal origin and related to manipulation of the gut[80,81]. However, this mechanism could hardly explain the elevation of pancreatic lipase.

The method of insertion and withdrawal of the endoscope might be a factor in the origin of post-DBE pancreatitis. However, in accordance with the literature[52], we did not find any differences in technique between our center and others that are performing DBE in Europe, and we did not see any differences even observing the masterful work of Professor Yamamoto during his live workshops at our endoscopy unit.

Differences in definitions of post-procedural complications offer a likely explanation for the difference in reported post-DBE pancreatitis[52].

In our opinion, mechanical stress on the pancreas seems possible, and the increased level of pancreatic lipase could be a correlate of this. We considered a possible influence of our learning curve on the incidence of hyperamylasemia in our patients, but we did not confirm this by subsequent analysis. The second point of our reflection was the type of endoscope. We used the thicker and stiffer EN-450T5 endoscope, so we were afraid of more forceful pressing of this device onto the pancreas. However, three cases of acute pancreatitis after peroral DBE using the EN-450P5 have been reported in the Netherlands[14] and one after DBE using the EN-450T5[66], and there were no differences in the type of endoscope in large studies[38].

Another question is prevention of post-DBE pancreatitis. We use parenteral hydration during after the oral procedure. The usual dose is 1 L of saline solution during a 2-h procedure. We believe that hydration could improve blood supply to the splanchnic region, pancreatic microcirculation, and post-procedure recovery. The use of proteinase inhibitors such as gabexate mesylate for the prevention of post-endoscopic pancreatitis has been disappointing[53,82]. There have been some studies of intravenous nitroglycerin[83], ulinastatin[84], somatostatin[53,82,85,86], rectal diclofenac[84], and other drugs for the prevention of post-procedure pancreatitis, but the results have not been significant.

CONCLUSION

Acute pancreatitis is a feared complication of oral DBE (51 cases of acute pancreatitis have been described in the literature to date, one of them fatal). Acute pancreatitis is the most common complication seen after diagnostic oral DBE (complications of therapy itself prevail in therapeutic procedures). Hyperamylasemia and elevation of pancreatic lipase after DBE seems to be a common occurrence. Association with acute pancreatitis is possible, but not obligatory. The diagnosis of acute pancreatitis is complex. It can be suspected clinically, but requires biochemical, radiological, and sometimes histological evidence to confirm the diagnosis. The complication rate of acute pancreatitis is reported at about 0.3% of DBEs according to large studies, almost solely after oral DBE. Drawbacks and possible bias of those studies are that they were mostly retrospective, a substantial number of DBEs were performed on an outpatient basis, and the follow-up was inadequate. Nowadays, it is clear that the oral DBE procedure is of higher risk in comparison with the anal one. It would be more precise to count the pancreatitis risk from oral procedures separately. By including anal procedures in determination of post-DBE pancreatitis rate, we obtain much lower and biased numbers. The presumable number of cases of acute pancreatitis after oral DBE is 1.5%-2%. In all patients with abdominal pain during the procedure and/or after oral DBE, diagnosis of acute pancreatitis should be considered and treatment should be provided in good time, the same as in post-ERCP pancreatitis. From the results of our study, we established the following rules in our clinical practice. Conscious sedation seems to be more favorable than general anesthesia due to monitor-

ing of the patient's pain during the procedure. Intense pain during the procedure may be a sign of inadequate pressure on the pancreas and pose a high risk of post-DBE pancreatitis. CO_2 insufflation during DBE is highly recommended as it prevents over-inflation of the small bowel, however, a possible preventive relationship to post-DBE pancreatitis has not been determined yet.

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REVIEW

Progress in laparoscopic anatomy research: A review of the Chinese literature

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Abstract

The development of laparoscopic surgery has generated the new field of study, laparoscopic anatomy. This article reviews the reported literature on laparoscopic anatomy and explores how it has evolved along with advances in abdominal surgery. In addition, the principal concerns in current laparoscopic anatomy research are discussed, including: (1) types of special adjacent anatomical structures; and (2) special surgical planes and anatomical landmarks. Understanding of systematic laparoscopic anatomy can provide the junior surgeons a clear procedural approach, and would benefit laparoscopic surgeons in training.

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Key words: Anatomy; Landmark; Laparoscopy; Minimally invasive; Surgical plane

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INTRODUCTION

The current surgery relies heavily on the use of laparoscopy due to its minimally invasive nature. In the field of abdominal surgery, the traditional approach of open surgery and related surgical anatomy are well established, while laparoscopic surgery and correlative laparoscopic anatomy are still under study.

Since laparoscopic surgery produces less trauma, and yields rapid recovery and superior cosmetic effects, it has become increasingly popular among both patients and physicians. However, laparoscopic surgery is not yet advanced enough to overcome its disadvantage of the lack of tactile sense. This impedes the sensitivity of applied laparoscopy to define the important anatomic features, structure locations, and boundary and texture of excision target tissues. Hand-assisted laparoscopy was developed to give surgeons more control and sensitivity while using laparoscopic instrumentation and digital visualization tools, which has been applied widely in abdominal surgery. Unfortunately, the lap-disc sealing and access device is costly and not extensively used in operations.

A second compelling disadvantage in the laparoscopic procedure is the loss of hand flexibility of the surgeon due to limited availability of space in the patient's body, thus increasing the technical difficulty of the procedure.

However, a systematical knowledge of laparoscopic anatomy can help surgeons to address this challenge. Clarified anatomical structures in the high-resolution and high-magnified view are of great significance in miniscule anatomical procedures, with little bleeding in the laparoscopic field of sight in the correct surgical plane while dissection was performed. Thus, gaining a thorough understanding of the laparoscopic anatomy is crucial to a successful laparoscopic surgery.

In order to provide guidance for laparoscopic surgeons in anatomy and technique, we have reviewed and summarized the current laparoscopic anatomical studies on abdominal surgery published in the literature. Although there are variations in anatomy among individual patients and in procedures used among surgeons, many commonalities exist and can be generalized to aid in the application of laparoscopy.

Systematic literature search was performed on PubMed, Medline, ScienceDirect, SpringerLink, CMCI, VIP, and CNKL to find relevant articles using the terms "laparoscopy", "anatomy", and "minimally invasive". Papers both in English and Chinese concerning anatomical characteristics in laparoscopic procedures in the fields of abdominal surgery were identified. The search results were classified as: (1) anatomical descriptions; (2) anatomical variations; (3) surgical planes and landmarks; and (4) measurements of certain structures. Papers dealing with different types of laparoscopic procedures were summarized according to the above classifications to identify the key points of each procedure.

LAPAROSCOPIC CHOLECYSTECTOMY

Studies on laparoscopic anatomy of laparoscopic cholecystectomy (LC) mainly focused on Calot's triangle, including the cystic artery and cystic duct[1,2]. The cystic artery normally has 1-3 branches into the gallbladder, originates from the proper hepatic artery, and is located within Calot's triangle. The variation of the common structure included origination from the right hepatic artery (Figure 1A), or from gallbladder bed of an aberrant type. The cystic duct is normally connected directly to the right wall of the hepatic duct; variations included connection to the left wall of the hepatic duct through an anterior or posterior approach (Figure 1B). Other structural differences reported in the literature were as follows: cystic duct location in parallel with the common hepatic duct (Figure 1C), presentation as an aberrant bile duct, adhesion and atresia, and an even connection to the right hepatic duct (Figure 1D).

Anatomical landmarks in the LC were mainly reported as Rouviere's sulcus, cystic lymph nodes and arteries[3]. The Rouviere's sulcus is a useful demarcation for the division between the S5 and S6 of the liver. When the gallbladder ampulla is stretched to the head side, the Rouviere's sulcus can be exposed. The Rouviere's sulcus was reported as an accurate landmark for the common hepatic duct plane, as they are on the same transversal level, above which the dissection of Calot's triangle is safe. When the fascial strip in Calot's triangle was flattened, the cystic artery could be identified by its visible pulsation and the presence of an adjacent, cystic lymph node. The cystic artery has been reported to run through the level of the gallbladder neck; exposure of the cystic artery may help surgeons locate the gallbladder neck at the same level. In addition, identification of the cystic lymph node may help define the cystic duct and the cystic artery structures (Figure 1E).

The key concerns in LC anatomy research are how to protect the common bile duct and right hepatic duct, which are injury-prone during the laparoscopic procedure. It is necessary to clarify the anatomy of Calot's triangle and ensure that the superior Rouviere's sulcus is on a safe surgical plane.

LAPAROSCOPIC INGUINAL HERNIORRHAPHY

Studies on the laparoscopic anatomy of herniorrhaphy focused primarily on inguinal hernias. Currently, there are two main approaches for laparoscopic inguinal herniorrhaphy (LIH), i.e. the transabdominal pre-peritoneal and the totally extra-peritoneal. Using laparoscopy, the presence of a direct hernia, indirect hernia or femorocele is readily found according to the position of the hernial ring, as shown in Figure 2A. There are marked differences in anatomical structure between oblique hernia and direct hernia. Thus, the size of mesh used in the operation should be decided according to each individual patient's anatomic data[4].

In the reported LIH procedures, meshes were always secured by either staples or sutures. Three potentially hazardous regions (Figure 2B) were identified and were seriously considered. (1) The so-called triangle of doom, wherein the medial margin is the deferent duct and the lateral margin contains the funicular vessels, should not be subject to staples or sutures because the iliac artery resides in this region; (2) An unexpected source of hemorrhage is identified as the corona mortis (translated as the crown of death), wherein lies the aberrant obturator artery and vein. Branches and distribution of the vascular connection between the obturator system and the external iliac or inferior epigastric systems, are located over the superior pubic ramus[5]; and (3) The triangle of pain, wherein the superior margin is the iliopubic tract (inguinal ligament), and the medial margin is the funicular vessel, is the region where the femoral branch of the genitofemoral nerve and the lateral femoral cutaneous nerve reside. The occurrence of post-operative pain was reported to have originated from staples or sutures placed in this region. It is reported that 15% of the lateral femoral cutaneous nerve was within 0.5 cm of the iliopubic tract or in the vertical plane of the anterior superior iliac spine[6]. To avoid post-operative pain, some surgeons opted against all use of the stapler.

Methods for distinguishing these important structures

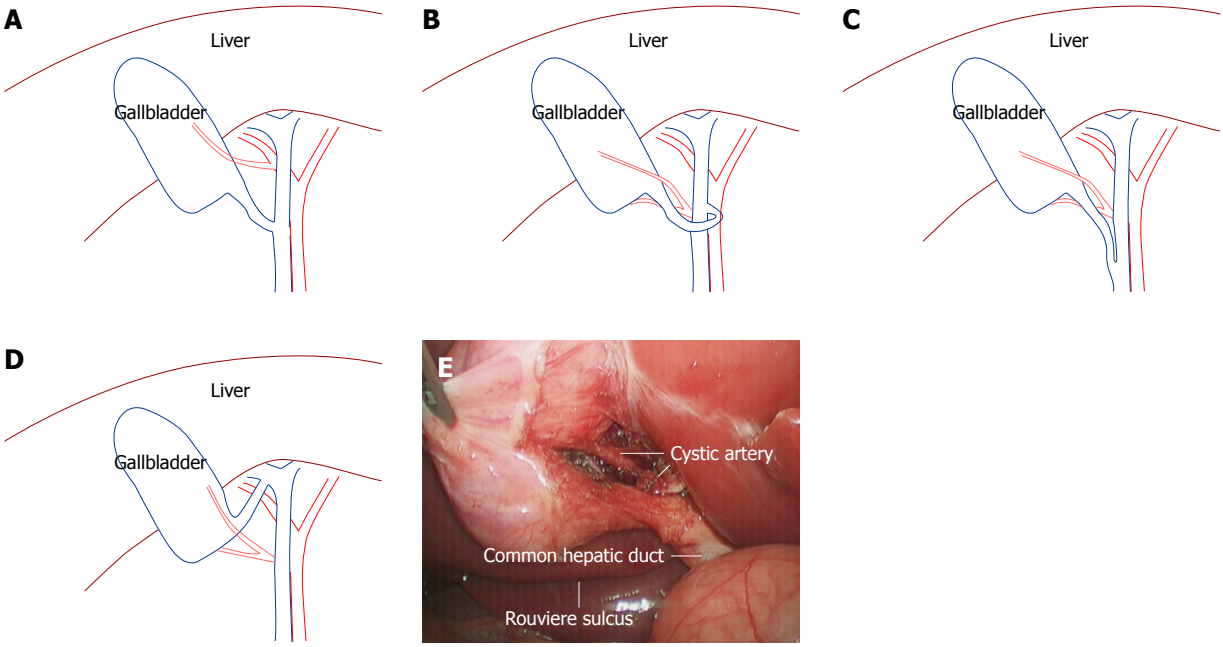


Figure 1 Anatomy of laparoscopic cholecystectomy (LC) (blue curves stand for bile duct and gallbladder and red curves for arteries). A: Illustration of a variant cystic artery originating from the right hepatic artery; B: Cystic duct connected to the left wall of hepatic duct *via* an anterior approach; C: Cystic duct parallel with common hepatic duct; D: Cystic duct connected to the right hepatic duct; E: Calot's triangle and Rouviere sulcus in LC.

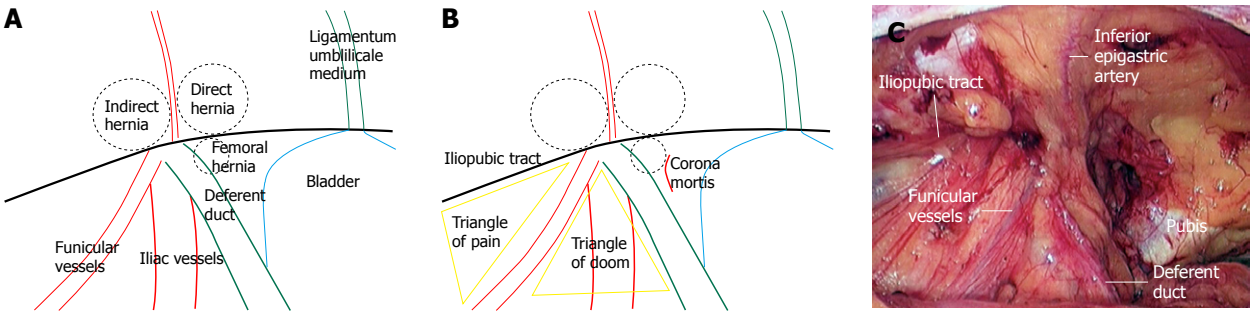


Figure 2 Illustration of inguinal hernia structures. A: Direct hernia was located medially to the inferior epigastric artery, an indirect hernia was located laterally to the inferior epigastric artery, and a femoral hernia was located at the inferior iliopubic tract; B: The three regions, where staples or sutures should not be placed: the triangle of doom, triangle of pain, and corona mortis; C: Live procedure after the peritoneum was absolutely dissected and removed from the abdominal wall.

are a main focus throughout the laparoscopic anatomy literature[7-9]. In live procedures, the structures could be totally exposed after the peritoneum was completely removed from the abdominal wall, as shown in Figure 2C.

The obvious hazards in LIH are the potential for injury of the deferent duct and unexpected hemorrhea. The major technique to protect the deferent duct in this procedure is skeletonization of the spermatic cord. In a nutshell, surgeons should take heed of one "cord", one "crown" and two "triangles".

LAPAROSCOPIC COLORECTAL RESECTION

Studies on the laparoscopic anatomy of colonic resection have focused on the left colon and rectum. Locating the root of the inferior mesenteric artery (IMA) and protecting the autonomic nerve and ureter are of importance in total mesorectal excision. The reports identified a particular landmark and a surgical plane.

IMA is located between the horizontal portion of the duodenum and the bifurcation of the abdominal aorta; the pulsation of the aorta made the identification easy. The landmark used to find the root of IMA was the convex stalk on the surface of the abdominal aorta. The superior sigmoid colon mesentery was lifted to the left side, straightening out the IMA to form an included angle, and the margins were defined as the abdominal aorta and the root of IMA[10] (Figure 3).

The important surgical plane that is yet to be defined is known as Toldt's space. During embryonic development, the mid-gut mesentery after succeeding in the volvulus combined with the peritoneal wall layer to form Toldt's fascia. Toldt's space appeared and remained consistently between the lateral left mesocolon and pre-renal fascia, as well as between the medial left mesocolon and

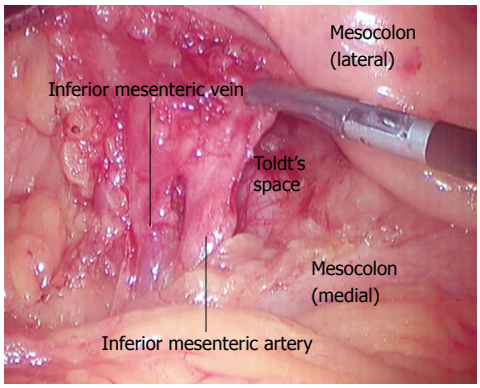


Figure 3 The root of inferior mesenteric artery. The inferior mesenteric artery and vein were identified when the superior sigmoid colon mesentery was lifted to the left side. Toldt's space was found in the included angle of the inferior mesenteric artery and abdominal aorta.

pre-aortic fascia. The space also appeared between the mesorectum and parietal layer of the pelvic fascia.

On the medial side, Toldt's space in the angle of the abdominal aorta and IMA was a vessel free area (Figure 3). IMA ran along the surface of the cephalic abdominal aorta, down to the pelvic cavity and pre-sacral space[11,12]. On the lateral side, an obvious yellow-white borderline was visible. The yellow portion is a part of the sigmoid mesocolon, and the white is a lateral abdominal wall. Dissecting from the medial and lateral space frees the colorectal mesentery completely. The surgical planes are between the colorectal mesenteries and the continuous pre-renal fascia.

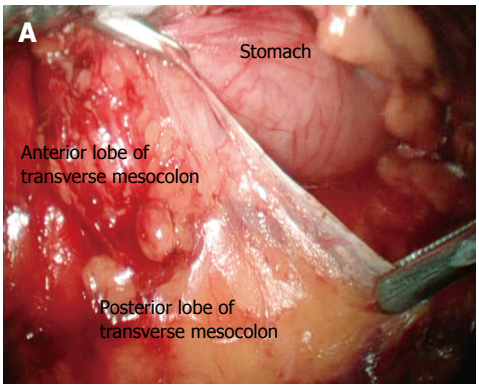
Dissecting in the Toldt's space might protect autonomic nerves and the ureter because they are all located beneath the pre-renal fascia. The superior hypogastric plexus closely associates with the back of the IMA. No obvious branches of autonomic nerves were found in the loose space between the visceral layer fascia (mesorectum) and the wall layer fascia (parietal pelvic fascia) at the back of the rectum[13].

In summary, the root of IMA and Toldt's space, which guided most total dissections of lymph nodes and protected the autonomic nerve, appear to be the most important anatomic structures in laparoscopic colorectal resection.

LAPAROSCOPIC GASTRECTOMY

Laparoscopy was often used to treat benign gastric diseases during the early stages. Recently, laparoscopic surgery has been increasingly used in radical gastrectomy, and more researches in laparoscopic anatomy have appeared[14,15], focusing on identifying crucial surgical planes and anatomical landmarks.

In LRG, there appeared to be two surgical planes that were important to define. The first was found between the posterior layer of the dorsal mesogastrium and the pancreas. The derivation of the posterior layer of the dorsal mesogastrium formed the gastrosplenic ligament, pancreatic fascia, pancreaticoduodenal fascia and the anterior lobe of the transverse mesocolon, which was continuous with each other as integrity. The surgical plane could be fixed in the space between the anterior and posterior lobe of the transverse mesocolon (Figure 4A). The plane could be reached more easily for dissection from the shortest distance of connecting fascia from the transverse colon to the descending part of the duodenum. The second surgical plane was identified in the space between the retropancreatic and pre-renal fascia, a safe plane for retropancreatic lymph node dissection. The pre-renal fascia was the post-boundary for ensuring a safe operational plane.



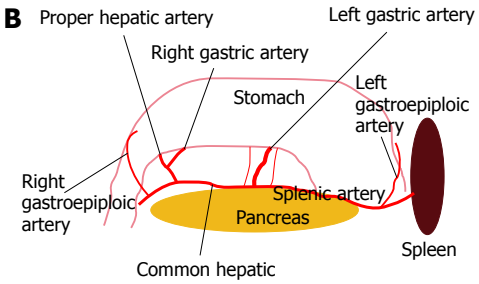


Figure 4 Anatomy of LRG. A: Dissection in the first surgical plane, i.e. the space between anterior and posterior lobes of the transverse mesocolon; B: The main vessels in the space of the posterior layer of dorsal mesogastrium and pancreas when the paries posterior gastricus was turned over to the head side.

Because of restricted laparoscopic sight and instrument manipulability, the procedure was performed from the caudal end to head. Therefore, the main vessels were identified in the space of the first surgical plane mentioned above. The paries posterior gastricus was gradually turned over to the head side following the opening of the first surgical plane, where each important vessel could be identified with the guidance of the following landmarks (Figure 4B). The pancreas was the largest central landmark. When dissecting in the gastropancreatic ligament near the pancreas head, the gastroduodenal artery and right gastroepiploic artery could be identified. Likewise, when dissecting in the splenocolic ligament near the pancreas tail, the left gastroepiploic vessels could be exposed. After the pre-pancreas fascia was absolutely dissociated, two folds were formed by the pre-pancreas fascia and its related vessels. The hepatopancreatic fold was the landmark used to find the common hepatic artery and proper hepatic artery at the inferior

margin of the pancreatic neck. The gastropancreatic fold was used to locate the left gastric artery.

In LRG, lymph nodes dissection was complicated, which could be achieved by recognizing and skeletonizing the related vessels. In summary, one center (pancreas), two ligaments (gastropancreatic and plenocolic ligaments), and two folds (hepatopancreatic and gastropancreatic folds) are the key landmarks identified in the literature used to find each vessel. These landmarks could be readily defined if the surgical plane (space between posterior layer of dorsal mesogastrium and pancreas) was appropriately identified.

OTHER LAPAROSCOPIC PROCEDURES

Anatomic studies of laparoscopic liver resection were mainly carried out by autopsy. The research priorities were placed on the first and the secundum porta hepatis[16,17]. Laparoscopic procedures were mostly performed as hepatic left lateral lobectomies. Therefore, the important structure of the left triangle ligament was defined as the peritoneal reflection of the diaphragm and the left lateral lobe. Superior hepatic arteries, branches originating from the left gastric artery and inferior phrenic artery, could be found after the left triangle ligament was opened (taking up 80%). The trunk of the superior hepatic arteries crossing the left hepatic veins of the superior posterior margin was an important landmark for locating the hepatic vein, as well as the secundum porta hepatis[18]. The falciform ligament was found as the anterior pathway to reach the secundum porta hepatis by laparoscopy. The left, inferior phrenic artery and middle segment artery of the liver formed a vessel that arched and gave off 6-12 branches to the falciform ligament, finally draining into the left inferior phrenic vein. The falciform ligament provides significant collateral circulation to the liver and is an important landmark for laparoscopic liver surgery[19].

Laparoscopic pancreaticoduodenectomy (LPD) is recognized as an extremely difficult laparoscopic operation due to the emergency status of the laparoscopic surgery. Chinese surgeons[20] developed a method of retroperitoneal laparoscopic dissection to expose the pancreatic body and tail. The retro-pancreatic space was reached using testis (ovarian) vessels as anatomical landmarks. The peritoneum was a landmark for the belly side of the para-renal space, and the splenic artery was a landmark of the upper pancreatic edge. The left renal vein was a landmark of the lower pancreatic edge, the left diaphragm colon ligament (or splenic lower pole) was a landmark for the pancreatic tail, and the intersection point between the right edge of the inferior mesenteric vein and the inferior margin of the pancreas were landmarks for the transition part of the pancreatic neck and body. Lu *et al*[21] developed the duodenal wall approach, in which the lateral wall of the duodenum was dissected and the root of the mesenteric vessel could be seen at the horizontal level of the duodenum; and the posterior pancreatic segment of the visible portal vein could be located by searching along the superior mesenteric vein.

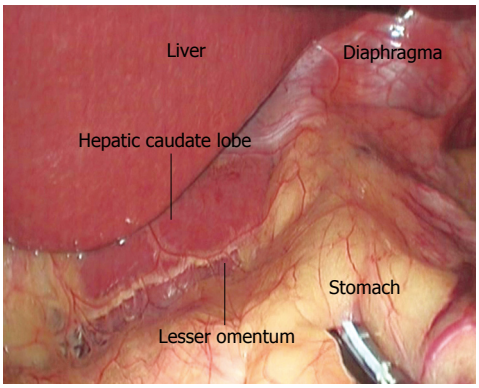


Figure 5 The lesser omentum above the hepatic caudate lobe.

Laparoscopic fundoplication, routinely used in laparoscopic anti-reflux surgery (LS), is a classical approach for the treatment of gastroesophageal reflux disease. Its related laparoscopic anatomy emphasized the anatomic landmark and surgical plane. The procedure is initiated by opening the lesser omentum above the hepatic caudate lobe, the landmark for finding the right crura of diaphragma. The space between the right crura of diaphragma and the esophagus is the surgical plane for dissociating the esophagus[22] (Figure 5). Special anatomical variations, such as an aberrant left hepatic artery, can complicate the procedure[23].

Anatomic studies on laparoscopic splenectomy focused on the splenic vessels. In most cases, splenic arteries and veins run together along the superior edge of the pancreatic body and tail; the arteries are located above the veins. In some reported cases, the splenic arteries ran along the superior edge of the pancreatic body tail, and the splenic arteries and veins were either found at the back of the pancreas, or the arteries were absolutely absent around the pancreatic body and tail. It was required that two (in most cases), three, and four splenic, lobar branches should be clamped[24,25].

CONCLUSION

What can we learn from the entire body of literature on laparoscopic anatomy?

Researches on laparoscopic anatomy have not been extensively carried out or reported throughout the world. Perusal and a summary of all of the relevant studies indicated that China-based scholars did most of the research work on laparoscopic anatomy. Some works were initially carried out by autopsy, and the results were provided to surgeons for guidance in live procedures. Procedures that were already extraordinarily familiar by surgeons, such as LC and LIH, were initiated in live surgeries.

Studies on the laparoscopic anatomy of abdominal surgery mainly focused on the following aspects: (1)

Simulating laparoscopic surgery on cadavers, or relevantly studying endoscopic anatomy through clinical laparoscopic surgery; (2) Obtaining relevant data by measuring the length, distance, and diameter of the tissue under the study; (3) Calculating the case percentage of special adjacent anatomical structures; and (4) Finding relevant anatomical planes, landmarks and providing relevant intraoperative pictures.

Due to the various surgical approaches, the portion of our study that focused on certain laparoscopic anatomy also varied. During laparoscopic procedure, the size and boundary of target tissues and the distance between two structures in laparoscopy sight were estimated by surgeons based on their experience. These data could not be obtained accurately by the naked eye using the existing laparoscopic technology. Therefore, some data in the autopsy studies could only be used as a reference during live laparoscopic procedures. The key point for laparoscopic surgeons was to understand the special, adjacent anatomical structure and the location of the correct anatomical plane and anatomical landmark. Gaining a definite understanding will guide future prospective studies of laparoscopic anatomy.

Surgical planes and anatomic landmarks

Why could there be less bleeding in the laparoscopic sight if manipulation occurs in certain surgical planes? Each surgical plane and natural space in the human body, are formed by stratum membranosum, like the membranous layer of the superficial fascia present in many regions of the body[26]. The basic research on surgical planes in laparoscopic anatomy should focus on vessel-less stratum membranosum.

Anatomical landmarks are descriptions of neighboring structures crucial to identifying the proper target tissue for resection. Although individual patients vary in their anatomical structure, certain commonalities exist. These commonalities become obvious through the numerous cases and procedures reported. Laparoscopic surgeons must rely on landmarks in each procedure; it is crucial in laparoscopy that detours must be minimized, otherwise an unexpected injury is likely to occur.

Sometimes, the surgical planes and anatomical landmarks can assist each other. In an appropriate plane, it would be easy to identify certain landmarks. Take LRG for example; if the space between the posterior layer of dorsal mesogastrium and pancreas could be found correctly, the landmarks of one center, two ligaments and two folds would be immediately obvious. Likewise, a landmark can help find readily a surgical plane. In LAS, the hepatic caudate lobe was the landmark for finding the right crura of diaphragma, the surgical plane for dissociating the esophagus.

Significance and prospective

Currently, laparoscopy is usually performed using the approach of natural orifice transluminal endoscopic surgery and a single entry port. These techniques require advanced skills of surgeons and a detailed knowledge of laparoscopic anatomy.

In the field of abdominal surgery, laparoscopic surgery has become increasingly popular. Systematic laparoscopic anatomy can provide laparoscopic surgeons with a clear idea to design their operation, and is particularly important for practicing novices. Laparoscopic anatomy is a basic curriculum for laparoscopic surgeons, and will certainly become an important new subject as the use of laparoscopic procedures increases.

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REVIEW

Circulating microRNAs: Novel biomarkers for esophageal cancer

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Abstract

Esophageal carcinogenesis is a multi-stage process, involving a variety of changes in gene expression and physiological structure change. MicroRNAs (miRNAs) are a class of small non-coding endogenous RNA molecules. Recent innovation in miRNAs profiling technology have shed new light on the pathology of esophageal carcinoma (EC), and also heralded great potential for exploring novel biomarkers for both EC diagnosis and treatment. Frequent dysregulation of miRNA in malignancy highlights the study of molecular factors upstream of gene expression following the extensive investigation on elucidating the important role of miRNA in carcinogenesis. We herein present a thorough review of the role of miRNAs in EC, addressing miRNA functions, their putative role as oncogenes or tumor suppressors and their potential target genes. The recent progresses in discovering the quantifiable circulating cancer-associated miRNAs indicate the potential clinical use of miRNAs as novel minimally invasive biomarkers for EC and other cancers. We also discuss the potential role of miRNAs in detection, screening and surveillance of EC as miRNAs can be a potential target in personalized treatment of EC.

INTRODUCTION

Esophageal cancer (EC) is the eighth most common cancer and the sixth most common cause of cancer death worldwide, affecting more male than female[1]. The dramatic geographic difference in incidence is the striking characteristic for EC. The EC incidence in the high-risk northern Chinese exceeds 100/100000, while that in the low-risk western Africa can be 20-fold lower[2]. EC remains the leading cause of cancer-related death in the high-risk areas, especially in Henan Province, northern China[3]. Esophageal squamous cell carcinoma (ESCC) and adenocarcinoma (EAC) are the two main forms of EC; each of them has different etiologic and pathologic characteristics. ESCC remains the predominant subtype of EC, especially in China; in contrast, EAC is the most common type in Western countries.

The survival and prognosis of EC patients depend on the stage of the tumor at the time of detection. Despite significant investment and advances in the treatment of cancer, the overall survival for advanced and metastatic cancer is still dismal, with a 5-year survival rate of less than 15% for advanced tumors[1,4,5]. The concept of early detection of esophageal cancer has been established

among clinicians and scientists for years. The 5-year survival rate was more than 90% for early EC[1,4,5], about 50% of cancers extend beyond the primary local region at the time of diagnosis and almost 75% of surgically treated patients have proximal lymph node metastasis[6]. At present, gastrointestinal endoscopy remains the primary screening tool, by which the suspected lesions can be biopsied for histopathological analysis. This invasive test, even though it is proved to increase the detection of early tumor and therefore can prolong the survival of the patient, is generally considered to be inconvenient and painful. Because of its limitation, there is an urgent request for discovery of novel predictive markers for EC.

Micro ribonucleic acids (miRNAs) are a class of single-stranded, evolutionarily conserved non-coding RNAs, only 17-25 ribonucleotides long[7]. The first known microRNA (miRNA), the lin-4, was discovered in 1993 through the study of heterochronic gene lin-14 in worms[8]. Since then, miRNA has shown the great potential regulating roles in many aspects of biological and pathological processes. The miRBase, which is the international registries for miRNAs, clearly described their nomenclature, targets and functions and implications in different diseases[9]. The biogenesis of miRNAs is a multi-step process involving a complex protein system, which includes members of the Argonaute family, Pol Ⅱ-dependent transcription and the RNase Ⅲs Drosha and Dicer[10]. MiRNAs are involved in crucial biological processes, including development, differentiation, apoptosis and proliferation[11,12]. The mechanism is still under debate, but probably related to inhibition of translation and messenger RNA degradation[13]. These small RNAs regulate gene expression at the level of translation through imperfect pairing with target messenger RNAs (mRNAs) of protein-coding genes[10]. The specific region in miRNA, which is important for messenger RNA target recognition, is referred to as the "seed sequence", and located at the 5- end of the mature miRNA sequence, from bases 2 to 8[11]. With the seed sequence, we can search for the complementary sequences in the 3'-untranslated regions (3'-UTRs) of known genes that exhibit conservation across species[11]. In animals, most miRNAs are thought to form imperfect base pairs with their target mRNA(s) and these interaction sites are enriched in 3'-UTRs[11]. To date, in the human genome, over 700 mature miRNAs have been identified, and the number is still increasing with the future studies. Up to 1000 miRNAs have been predicated by the bioinformatics studies of the human genome[14].

EXPERIMENTAL TECHNIQUES FOR MIRNA ANALYSIS

It is necessary to explore effective tools for detection, quantification, and functional analysis of miRNAs. Oligonucleotide miRNA microarray analysis has been extensively used as high-throughput technique for the assessment of cancer-specific expression levels in a large number of samples[10,13,15]. Another method to gauge miRNA expression level includes utilizing a bead-based flow-cytometric technique by which hybridization occurs in solution and provides a high specificity for closely related miRNAs[16]. The miRNA microarray platform with locked nucleic acid-modified capture probes can improve probe thermostability and increase specificity, so that it can discriminate miRNAs with even single nucleotide differences[17]. Quantitative real-time polymerase chain reaction (PCR) have also been widely applied to miRNA research because of its cost-effectiveness, high throughput and superior detection of low-abundance species[18]. All of these technologies facilitating miRNAs expression profiling are essential for validation of microarray data.

It is also necessary to develop methods of manipulating miRNA expression for elucidating biological significance and manipulating miRNA expression. MiRNA inhibitors such as 2-O-Methyl antisense single-strand oligonucleotides and locked nucleic acid modified oligonucleotides, made the suppression of endogenous miRNA activity and its downstream effect on messenger RNA expression possible[19]. The latest improvement of miRNA inhibition could control miRNA expression *in vivo* by expressing decoy miRNA targets *via* lentiviral vectors[20], with it we can inhibit specific miRNA by expressing various miRNA inhibitors. MiRNA mimicry could be used to identify the cellular processes and phenotypic changes through transfecting specific miRNAs into cell lines[21]. Although these techniques are of different strength and weakness, the large size studies have shown common characteristics of miRNA deregulation in human cancers[13] and shed light on the application of miRNAs as novel biomarkers finally.

MIRNA AND EC

Early studies demonstrated the aberrant expression of miRNA in cancers and the fact that approximately half of miRNA genes are localized in cancer-associated genomic regions or in fragile sites[22] indicates the potential role of miRNAs as a promising class of oncogenes or tumor suppressor gene in human carcinogenesis. MiRNAs still largely intact in conventionally collected, formalin-fixed and paraffin-embedded tissues and even a modest number of miRNAs are adequate to discriminate human tumors according to the developmental lineage and differentiation state of the tumors[16]. Furthermore, compared with messenger RNA (mRNA) profiles, it is easier to use these miRNA expression profiles to classify poorly differentiated tumors[23]. Here, we will focus on the miRNA expression profiles of EC, especially ESCC, to reveal the oncogenic mechanism by miRNA mediated post-transcriptional pathway. We will first discuss the identification of EC specific miRNA signatures for a new class of screening, diagnostic and prognostic biomarkers through miRNA expression profiling of EC; then we will concentrate on how to clarify the functional roles of miRNA in EC and the potential clinical valuation of the circulating miRNAs in EC. We believe that the study of miRNA's role in EC will provide new sight into this disease.

Table 1 Differential expression of miRNAs in EC tissues compared with normal tissue as identified in seven studies

Techniques	Pathology	Population	Overexpressed miRNAs			Underexpressed miRNAs		Ref.
Array profiling	ESCC	Chinese	hsa-miR-25	hsa-miR-424	has-miR-151	hsa-miR-100 hsa-miR-29c	hsa-miR-99a mmu-miR-140	[6]
mirVana miRNA Bioarrays (Ambion)	EAC	Japanese	miR-192 miR-21	miR-194 miR-93	miR-200c	miR-205	miR-203	[25]
	ESCC	Japanese	miR-342	miR-21	miR-93	miR-205	miR-203	
qRT-PCR	ESCC	Cell lines, Chinese	miR let-7d miR-373	miR-330	miR-340			[26]
qRT-PCR	ESCC	Japanese	miR-9 miR-17-5p miR-21 miR-34c miR-107 miR-130a miR-134 miR-151	miR-15b miR-20a miR-25 miR-103 miR-127 miR-130b miR-137	miR-16 miR-20b miR-34b miR-106a miR-129 miR-132 miR-138	miR-133a miR-139	miR-133b miR-145	[27]
Agilent Human miRNA microarray kit version 1.0	EAC	Caucasian	hsa-miR-126 hsa-miR-146a hsa-miR-195 hsa-miR-199a hsa-miR-424	hsa-miR-143 hsa-miR-181a hsa-miR-28 hsa-miR-30a-5p	hsa-miR-145 hsa-miR-181b hsa-miR-199b hsa-miR-29c	hsa-miR-149 hsa-miR-205 hsa-miR-221 hsa-miR494 hsamiR-617	hsa-miR-203 hsa-miR-210 hsa-miR-27b hsa-miR-513 hsa-miR-99a	[28]
miRNA microarray chips version 3	EAC	Caucasian	miR-21 miR-194	miR-192 miR-223		miR-203		[29]
	ESCC	Japanese	miR-21			miR-375		
qRT-PCR	ESCC	Cell lines, Chinese	miR-10					[30]

miRNAs: Micro ribonucleic acids; EC: Esophageal cancer; ESCC: Esophageal squamous cell carcinoma; EAC: Adenocarcinoma; qRT-PCR: Quantitative real-time polymerase chain reaction.

Role of miRNAs in carcinogenesis, progression, invasion and metastasis of EC

MiRNA acts as oncomiRNA or tumor suppressors to affect the tumorigenesis if their target mRNAs were encoded by tumor suppressor genes or ontogenesis[24]. Aberrant miRNA expression has been identified and confirmed in EC (Table 1). Comparing the miRNAs expression in ESCC with EAC, we can find that only a few miRNAs expressions were the same in these two diseases, each of which has the different miRNAs expression profiling, indicating that both of ESCC and EAC have distinct etiologic and pathologic characteristics. The miRNAs which are highly expressed in tumor can act as tumor promoter by targeting and inhibiting different tumor suppressor genes such as B-cell CLL/lymphoma 2 (BCL2)[31], *tension homologue* gene (PTEN)[32], large tumor suppressor homolog 2 (LAST2)[25], annexin A1 (ANXA1)[33]. Those genes were all studied in EC tissues and it was suggested that all of them were involved in carcinogenesis of esophagus. Those mature miRNAs with relatively lower expression in cancer may act as tumor suppressor miRNAs. The loss of such miRNAs may enhance the activity of oncogenes.

Apart from identifying different cancer-related miRNAs, efforts have been made to identify their target genes, messenger RNAs and receptors, which will lead to further studies about their contribution in cancer treatment. For example, the putatively identified targets[31,32,34] of miR-21 are BCL2, PTEN, tumor suppressor gene tropomyosin 1 (TPM1), programmed cell death 4 (PDCD4) and maspin. Zhu *et al*[34] found that the biological function of miR-21 is probably due to the simultaneous repression of multiple tumor suppressor genes, including PTEN, TPM1, PDCD4, and maspin. PTEN is capable of restricting growth and survival signals by limiting the activity of the phosphoinositide 3-kinase (PI3K)/AKT pathway[35]. A decrease in functional PTEN causes constitutive activation of downstream components of the PI3K/AKT pathway, leading to tumor progression and metastasis[35]. Thus, down-regulation of PTEN by miR-21 may contribute to transformation and increased tumor cell survival[35]. TPM1 is an actin-binding protein which is capable of stabilizing microfilaments, regulating both microfilament organization and anchorage-independent growth, so its importance in cell transformation has been highlighted[36]. Another important mechanism of cancer development is epigenetic silencing of tumor suppressor genes. DNA hypomethylation, CpG island hypermethylation and histone-modification losses represent epigenetic markers of malignant transformation[37]. Calin *et al*[22] showed that some miRNA (e.g. miR-127) are located in a CpG island, a region that is involved in several types of translocations identified in hematological cancers and deleted by loss of heterozygosity in solid tumors. This phenomenon gives us a clue that the expression of tumor suppressor can be altered in epigenetic silencing mechanism through expression of miRNA.

Dysplasia and Barrett's esophagus are precursors of EC and the progression of these lesions to cancer is a multistep process that involves different sequential DNA aberrations and changes in gene expression. MiRNA expression profiles in tissues of Barrett's esophagus with high-grade dysplasia were significantly different from

their corresponding normal tissues/EAC tissues, but, no significant difference was found between low-grade dysplasia and paired normal tissues[28]. These observations were consistent with the finding of Feber *et al*[25], which suggests that low-grade dysplasia was with a lower malignant transformation rate compared with high-grade dysplasia. Yang *et al*[28] found that seven miRNAs (hsa-let-7b, hsa-let-7a, hsalet-7c, hsa-let-7f, hsa-miR-345, hsa-miR-494, and hsa-miR-193a) were potentially important in the progression from high-grade dysplasia to EAC. Hsa-let-7b, hsa-let-7a, hsalet-7c and hsa-let-7f belong to the let-7 miRNA family, most of their members function as tumor suppressors through negative regulation of the *RAS* gene[38]. Accordingly, RAS mutations and amplifications have been frequently identified in precancerous tissues and EC tissues[39,40]. Oncogene RAS may play a role in cell growth and differentiation[41].

Diagnostic and prognostic value of miRNA for EC

To date, the available data of miRNA expression profile in cancer suggest that miRNA may have diagnostic and prognostic potential. It is crucial to correlate miRNA expression with tumor subtypes or clinical parameters.

Lu *et al*[16] were the first to systematically demonstrate that the specific miRNA expression can be applied not only for diagnosis but also for classification of different tumors, and the accuracy is 70%. Guo *et al*[6] were the first to investigate the expression of miRNA in EC tissues using miRNA microarray techniques. They found that five miRNAs (miR-335, miR-181d, miR-25, miR-7 and hsa-miR-495) correlate with gross pathologic classification (fungating *vs* medullary) and two miRNAs (miR-25 and miR-130b) correlate with differentiation classification (high *vs* middle *vs* low)[6]. The miRNA expression profiling of ESCC is distinct from EAC[25]. Compared with its expression in normal epithelium (NSE), miR-194, miR-192 and miR200c are significantly up-regulated in EAC but not in ESCC[27]. Oppositely, miR-342 is aberrantly expressed in ESCC but not in EAC[25]. Furthermore, the miRNA expression profiling of NSE and SCC samples are more similar to each other than to the EAC samples[26]. The Barrett's esophagus (BE) samples sit between the EAC and NSE and one high-grade dysplasia specimen has a miRNA expression profile similar to the EAC[24]. This makes good biological sense that miRNA expression profiles can distinguish esophageal tumorigenesis and may be proven useful for identifying BE patients who are at high risk for progression to EAC.

As we mentioned before, the survival and prognosis of EC is dismal. Therefore, the discovery of new prognostic markers could be a prodigious advantage for identification of patients that would benefit from more aggressive therapy. The high expression of both miR-103 and miR-107 has been shown to correlate with poor survival in 53 Chinese patients with ESCC either by univariate analysis or by multivariate analyses[6]. In an independent study of 30 Japanese patients with ESCC, overexpression of miR-129 was found to significantly correlate with a shorter survival and miR-129 was identified as a significant and independent prognostic factor in surgically treated ESCC patients[27]. The largest study so far, found that low expression of miR-375 in EAC patients was strongly associated with worse prognosis, whereas ESCC patients with high expression of miR-21 had poor prognosis[29]. An earlier study conducted by Sugito *et al*[42], measured the expression levels of DICER1, DGCR8, and RNASEN messenger RNA in 73 surgically treated ESCC patients by real-time reverse-transcription PCR, and clarified that high RNASEN expression correlate with poor prognosis in ESCC, indicating that RNASEN may be a good candidate for molecular prognostic marker. Altered expression of these miRNAs is tissue specific, which may provide clues as to the different molecular progression undertaken by these cancers, and opens a potential avenue for targeted therapy to improve prognosis[29].

Circulating microRNAs: Novel minimally invasive biomarkers for EC

Scientists studying on EC are continually dedicated to search for sensitive, and minimally invasive markers which can detect early neoplastic changes, thus identifying EC at an early stage, as well as monitoring the progress of patients with EC and their response to treatment. Existing methods for identification of EC have many inherent deficiencies. Endoscopic biopsy and histopathological examinations are currently the golden standard diagnostic methods for EC. However, it has limitations, including the use of invasive tool and limited number of experienced physicians. Besides, it is difficult to be applied in large-scale studies because of its tedious approaches. To date, there are still no other effective biomarkers established in the routine assessment of EC.

The much desirable biomarker should be sensitive enough and easily accessible to detect the cancer patients at early stage. Recent studies have shown that tumor-derived miRNAs are resistant to endogenous ribonuclease activity so it can be present in human serum in remarkably stable form[43]. Furthermore, the expression level of serum miRNAs is reproducible and consistent among individuals[43,44]. These tumor-derived miRNAs are present in the circulating blood at levels sufficient to be measurable as biomarkers for the detection of tumors and more than 100 circulating miRNAs can be detected in the blood of healthy individuals[43]. Either plasma or serum can be used for identification of these blood-based biomarkers because the miRNAs levels in plasma and serum are strongly correlated[43,45]. Chen *et al*[44] demonstrated that the colorectal cancer patients shared a large number of serum miRNAs with lung cancer patients. Moreover, they identified a unique expression profile of serum miRNAs for colorectal cancers that were not present in lung cancer. Though miRNA research is only emerging recently, it has aroused great interest in clinical and scientific communities. Since the presence of miRNA in serum was first described in patients with diffusive large B-cell lymphoma[46],

a number of studies on other solid cancers (ovarian cancer[47,48], lung cancer[49,50], breast cancer[51], colorectal cancer[52,53]) have been reported about the presence of miRNA in circulation and their potential for use as novel biomarkers. Taylor *et al*[48] demonstrated that the expression of 8 miRNAs in the serum of patients with ovarian cancer were significantly distinct from their expression observed in benign diseases, yet could not be detected in the serum of healthy controls. Another study indicated that miRNA levels were significantly different between lung cancer patients and controls[49]. Both of these studies suggested that there was no significant difference in the miRNA expression between peripheral circulation and tumors tissues[48,49], indicating that circulating miRNA could act as surrogate of tissue miRNA for early diagnosis. And this will initiate a new application that will be widespread in clinical treatment and disease prevention.

Blood-based miRNAs provide a novel class of minimal invasive biomarkers for high-risk subject screening and early diagnosis for cancers. At the time of writing, there was no article about circulating miRNAs profile of EC. We focus on the circulating miRNA expression profiles of ESCC which is the main type of EC, especially in Asia. Checking circulating miRNA expression profiles of ESCC can help identify EC specific circulating miRNAs signatures for a new brand of screening, diagnostic and prognostic biomarkers. Studies can also be performed to clarify the functional role(s) of miRNAs in EC.

Potential in screening, surveillance and treatment of EC

So far, endoscopic biopsy and histopathological examinations are the golden standard for high-risk subject screening and early detection for EC worldwide. We can only identify 1%-2% early carcinomas and 15%-20% precancerous lesions in asymptomatic population aged higher than 35 years in high-incidence areas of EC through endoscopic biopsy and histopathological examinations[3]. Yet nearly 80% asymptomatic populations were in the normal range. In other words, there exists over endoscopic application. Besides, as there are no sufficient experienced physicians in the countryside, the misdiagnosis and missed diagnosis would be inevitable. In addition, standard sterilizing procedure also restricts the wide application of endoscopy in asymptomatic population. Furthermore, the projected cost of mass screening in China for detection of cancers is increasing annually. An urgent and important problem to be solved is to diminish blindness in the screening, minify the range of endoscopy and elevate the detection sensitivity of early carcinoma in high risk subjects. Easily accessible and minimal invasive biomarkers for EC and for other diseases are much desirable. The PCR technique for detection and quantification of miRNA[18] in the blood would be one of the promising methods of screening individuals for EC and other cancers because of its universal application, convenient management, low cost and high sensitivity. Rouet *et al*[54] set up a model of qRT-PCR for serum RNA analysis, suggesting that its cost was only less than £1 for each clinical specimen when used in a group of 84 patients. Once the accuracy of detection is established, MiRNAs test should be introduced into the national EC and other disease screening programs. The best option might be that annual miRNA blood test in asymptomatic populations in high risk areas of EC, and then the patients with positive test result can choose endoscopic biopsy and histopathological examinations for final diagnosis.

Given the emerging evidence of miRNAs acting as oncogenic or tumor suppressor activities, it is important to seek approaches to interfere with the miRNA[55], and eventually, develop these as novel biomarkers into EC therapies. Scientists are making efforts to explore miRNA and utilize that in cancer. Meng *et al*[32] showed that miRNA expression profiles changed during treatment with gemcitabine, and the sensitivity of cholangiocarcinoma tumor cells to this chemotherapy *in vitro* are increasing with the modulation of some miRNAs. On the contrary, it could be feasible to introduce tumor suppressor miRNA which are tissue-specific to the target gene, and then it may help prevent further progression, or even shrink EC. Yet, recently identified genomic and proteomic biomarkers, tumor cell mutations and microsatellite instability cannot be recommended for clinical use because of insufficient available data[56]. Though recent studies on miRNA in EC are limited, the potential value of miRNAs as prognostic and predictive biomarkers in EC is elegantly highlighted and will be elucidated eventually. However, experimental miRNA therapy need to be validated comprehensively through studies involving different cohorts of patients before introduced into clinical practice.

CONCLUSION

Although much of the work on miRNA is still in its infancy, the previous studies have demonstrated the significant role(s) of miRNA in the initiation and progression of cancer and other diseases. Further exploration is required for better understanding their role in carcinogenesis of EC and for better application in the future. Notwithstanding there are still many questions to be answered about circulating miRNA, there is no doubt that further studies will lead to great progress in the future therapy of EC. The exploration of miRNA will improve our knowledge of the roles of these novel biomarkers, and further demonstrate their true potential in therapeutic methods.

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ORIGINAL ARTICLE

Butyrate stimulates IL-32α expression in human intestinal epithelial cell lines

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Abstract

AIM: To investigate the effects of butyrate on interleukin (IL)-32α expression in epithelial cell lines.

METHODS: The human intestinal epithelial cell lines HT-29, SW480, and T84 were used. Intracellular IL-32α was determined by Western blotting analyses. IL-32α mRNA expression was analyzed by real-time polymerase chain reaction.

RESULTS: Acetate and propionate had no effects on IL-32α mRNA expression. Butyrate significantly enhanced IL-32α expression in all cell lines. Butyrate also up-regulated IL-1β-induced IL-32α mRNA expression. Butyrate did not modulate the activation of phosphatidylinositol 3-kinase (PI3K), a mediator of IL-32α expression. Like butyrate, trichostatin A, a histone deacetylase inhibitor, also enhanced IL-1β-induced IL-32α mRNA expression.

CONCLUSION: Butyrate stimulated IL-32α expression in epithelial cell lines. An epigenetic mechanism, such as histone hyperacetylation, might be involved in the action of butyrate on IL-32α expression.

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Key words: Cytokine; Histone acetylation; Inflammatory bowel disease

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INTRODUCTION

Interleukin (IL)-32 was originally reported as natural killer (NK) transcript 4, and has been described as a cytokine produced mainly by T-lymphocytes, NK cells, epithelial cells, and blood monocytes[1-3]. The gene encoding IL-32 is located on human chromosome 16p13.3 and is organized into eight exons[4]. There are four splice variants, and IL-32α is reported as the most abundant transcript[5,6]. Recently, IL-32 was defined as a proinflammatory cytokine characterized by the stimulation of secretion of IL-1β, tumor necrosis factor (TNF)-α, IL-6,

and IL-8 *via* the activation of p38 mitogen-activated protein kinases (MAPKs), nuclear factor (NF)-κB and activating protein (AP)-1 signal transduction pathways[3,7]. Recently, we reported the overexpression of IL-32α in the inflamed mucosa of inflammatory bowel disease (IBD)[8]. *In vitro* experiments on human intestinal epithelial cell lines showed that IL-32α expression was induced by IL-1β, IFN-γ and TNF-α through the activation of Akt-phosphatidylinositol 3-kinase (PI3K)[8].

Dietary fiber (nonstarch polysaccharides) and resistant starch escape digestion in the upper gastrointestinal tract, and undergo anaerobic bacterial fermentation in the colon. This process produces short-chain fatty acids (SCFAs), predominantly acetate, propionate, and butyrate, as the major by-products[9,10]. The typical concentration of each SCFA has been reported as approximately 10-20 mmol/L[11]. SCFAs have been shown to have significant effects on the colonic epithelium both *in vivo* and *in vitro*. Butyrate serves as the primary energy source for the normal colonic epithelium[12], and stimulates the growth of the colonic mucosa[13]. However, in tumor cell lines, it induces apoptosis and inhibits cell growth[14]. Butyrate has also been reported to modulate gene transcription through the induction of histone hyperacetylation. Since butyrate inhibits histone deacetylation and induces histone hyperacetylation, it is categorized as a histone deacetylase (HDAC) inhibitor[15].

In the present study, we investigated the *in vitro* effects of the SCFAs on IL-32α expression in human intestinal epithelial cell lines.

MATERIALS AND METHODS

Reagents

Recombinant human IL-1β and TNF-α were purchased from R&D Systems (Minneapolis, MN, USA), and human IFN-γ was obtained from Pepro Tech (Rocky Hill, NJ, USA). SCFAs (sodium acetate, sodium propionate and sodium butyrate) were purchased from Sigma Chemical Co. (Dorset, UK). Biotinylated anti-human IL-32α antibodies were purchased from R&D Systems, and horseradish peroxidase (HRP)-conjugated streptavidin was purchased from Dako Japan (Kyoto, Japan). Antibodies against phosphorylated and total Akt were obtained from Cell Signaling Technology (Beverly, MA, USA). Trichostatin-A was purchased from Tocris Cookson (St. Louis, MO, USA).

Cells

The human intestinal epithelial cell lines HT-29[16], SW480[17], and T84[18] were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured as described previously[16-18].

Real-time polymerase chain reaction

Total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform method, and was then reverse-transcribed into cDNA using a PrimeScript RT reagent kit (TAKARA-BIO, Shiga, Japan). The expression of human IL-32α mRNA was assessed by real-time polymerase chain reaction (PCR) analyses. Real-time PCR was performed using a LightCycler 2.0 system (Roche Applied Science, Tokyo, Japan). The oligonucleotide primers used in this study were specific for human IL-32α as follows: 5'-AGCTGGAGGACGACTTCAAA [nucleotides 192-211, GenBank accession no. BC018782[19,20]] and 3'-AGGTGGTGTCAGTATCTTCA (nucleotides 642-623)]. The PCR was conducted using a SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). β-actin was used as an endogenous control to normalize for differences in the amount of total RNA in each sample.

Western blotting analysis

The intracellular expression of IL-32α protein was determined by Western blotting. Briefly, the cells were washed and lysed in sodium dodecyl sulphate (SDS) sample buffer containing 100 μmol/L orthovanadate. The lysates were homogenized, and the protein concentration was measured by the Bradford method. For Western blotting, 10 μg of protein from each sample was subjected to SDS-PAGE on a 4%-20% gradient gel under reducing conditions. The proteins were then electrophoretically transferred onto a nitrocellulose membrane, and the membrane was blocked with 5% skimmed milk. After washing with PBS containing 0.1% Tween-20 (PBST), the membrane was incubated with a biotinylated anti-human IL-32α antibody at 4℃ overnight. Next, the membrane was reacted with HRP-conjugated streptavidin at room temperature for 1 h. The detection was performed using enhanced chemiluminescence (ECL) Western blotting systems (Amersham Biosciences).

Detection of global histone H3 acetylation

Histone extraction and the detection of global histone H3 acetylation were performed using the Epiquik global histone H3 acetylation assay kit (Epigentek; Brooklyn, NY, USA). The histone protein content was detected by the Bradford method.

Statistical analysis

Statistical significance of the differences was determined using unpaired *t* test (Statview version 4.5). Differences resulting in *P* values less than 0.05 were considered to be statistically significant.

RESULTS

Effects of SCFAs on IL-32α mRNA expression

To evaluate the effects of SCFAs on IL-32α mRNA expression, intestinal epithelial cells (HT-29, Caco-2, T84 cells) were stimulated with 10 mmol/L of each SCFA (acetate, propionate, and butyrate) for 12 h, and then the expression of IL-32α mRNA was determined by real-time PCR. Acetate had no effect, but butyrate significantly increased IL-32α mRNA expression in all cell lines. Propionate increased the expression of IL-32α

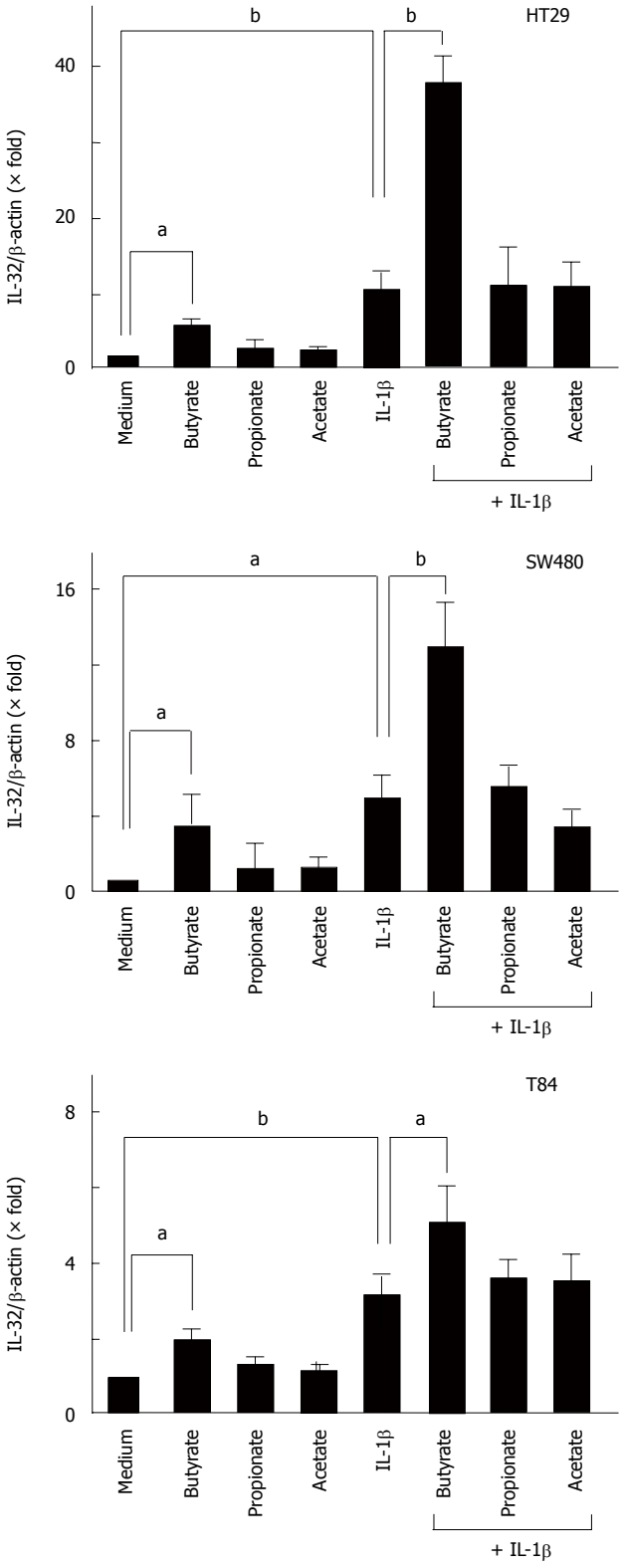


Figure 1 Effects of short-chain fatty acids (SCFAs) on IL-32α mRNA expression in colon cancer cell lines. The cell lines (HT-29, SW480, T84) were incubated for 12 h with each SCFA (10 mmol/L). In another experiment, the cells were stimulated with IL-1β (50 ng/mL) in the presence or absence of each SCFA (10 mmol/L). The IL-32α mRNA expression was determined by real-time PCR. The data are expressed as mean ± SD (n = 4). $^{a}P < 0.05$, $^{b}P < 0.01$.

mRNA only in T84 cells, but in the other cell lines it had no effect (Figure 1).

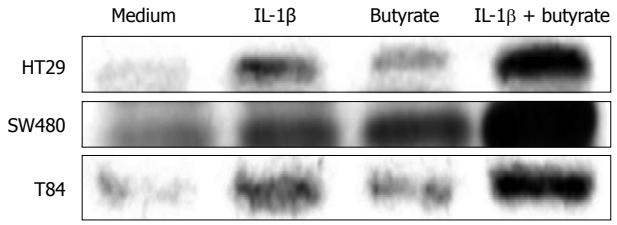


Figure 2 Effects of SCFAs on IL-32α protein secretion in colon cancer cell lines. The cell lines (HT-29, SW480, T84) were stimulated for 48 h with IL-1β (50 ng/mL) in the presence or absence of butyrate (10 mmol/L), and the intracellular IL-32α protein levels were detected by Western blotting.

Effects of SCFAs on cytokine-induced IL-32α mRNA expression

Next, we investigated the effects of SCFAs on cytokine-induced IL-32α mRNA expression. We recently reported that IL-1β, IFN-γ and TNF-α stimulate IL-32α mRNA expression in intestinal epithelial cells[8], and the cells were stimulated with cytokines in the presence or absence of each SCFA (10 mmol/L). In all cell lines, butyrate by itself stimulated IL-32α mRNA expression, and enhanced the IL-1β-induced IL-32α mRNA expression. SCFAs did not modulate IFN-γ- and TNF-α-induced IL-32α mRNA expression in any of the cell lines (data not shown).

Effects of SCFAs on intracellular IL-32α protein accumulation

To confirm the effects of SCFAs on IL-32α protein expression, the cells were stimulated for 48 h with IL-1β and/or SCFAs. Intracellular IL-32α was detected as a 25 kDa protein[8,21]. The effects of the SCFAs were similar to those observed at the mRNA level. The combination of IL-1β and butyrate enhanced IL-32α protein expression additively (Figure 2).

Dose responses

To examine the effects of butyrate on IL-1β-induced IL-32α expression more precisely, HT-29 cells were incubated for 12 h with IL-1β (50 ng/mL) plus increasing concentrations of butyrate. The effects of butyrate on IL-1β-induced IL-32α secretion appeared at the concentration of 5.0 mmol/L, and the most significant difference was observed at 10 mmol/L (Figure 3).

Effects of butyrate on the phosphorylation of Akt

We tested the effects of butyrate on the IL-1β-induced phosphorylation of Akt, which plays a major role in IL-32α induction[8,19]. As shown in Figure 4, there were no effects of butyrate on IL-1β-induced Akt phosphorylation. These observations suggest that the modulation of Akt activity is not involved in the actions of butyrate on IL-1β-induced IL-32α expression.

Effects of trichostatin-A on cytokine-induced IL-32α mRNA expression

Previous studies have demonstrated that butyrate exerts its biological effects through the induction of histone

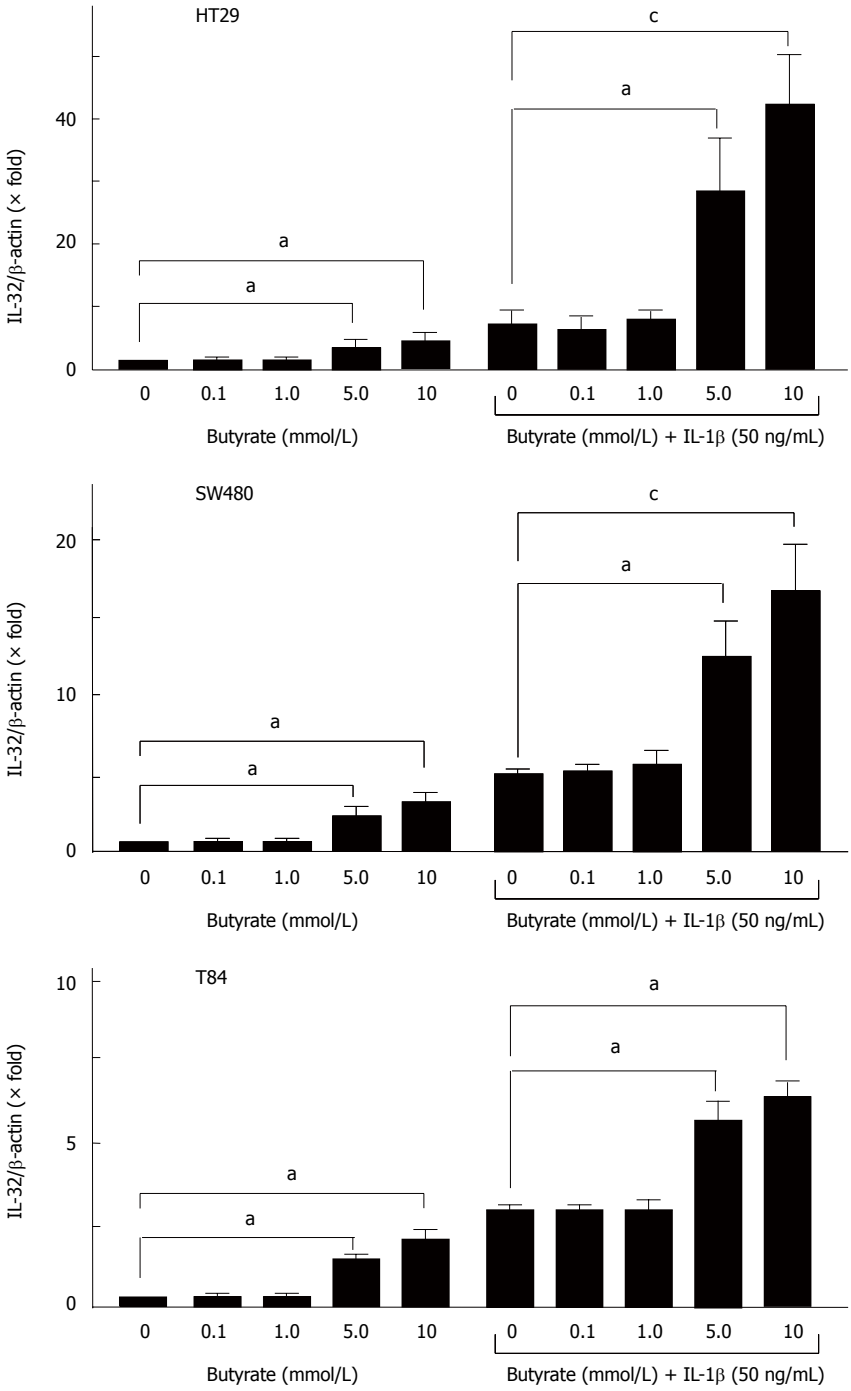


Figure 3 Dose-dependent effects of butyrate on IL-1β-induced IL-32α mRNA expression in colon cancer cell lines. The cell lines (HT-29, SW480, T84) were stimulated for 12 h with increasing concentrations (0-10 mmol/L) of butyrate in the presence or absence of IL-1β (50 ng/mL). IL-32α mRNA expression was then analyzed by real-time PCR. The data are expressed as mean ± SD (n = 4). [a]$P < 0.05$, [c]$P < 0.005$.

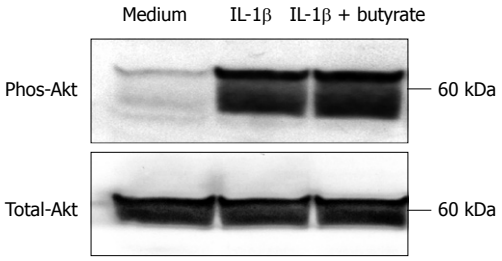


Figure 4 Effects of butyrate on Akt phosphorylation. The cells were stimulated in the presence of butyrate (5 mmol/L) and/or IL-1β (50 ng/mL) for 15 min, and then Akt phosphorylation was determined by Western blotting.

hyperacetylation[15]. To examine the relationship between histone acetylation and the effects of butyrate on IL-32α mRNA expression, we tested the effects of trichostatin-A (TSA), a potent histone deacetylase inhibitor[22-24].

The cells were stimulated with cytokines in the presence or absence of TSA (5 μmol/L), and the expression of IL-32α mRNA was then analyzed by real-time PCR. As shown in Figure 5, TSA enhanced the IL-1β-induced IL-32α mRNA expression in all cell lines.

Effects on histone H3 acetylation

We tested the effects of butyrate and IL-1β on histone H3 hyperacetylation in HT-29 cells. As shown in Figure 6, butyrate induced histone H3 hyperacetylation in HT-29 cells (Figure 6).

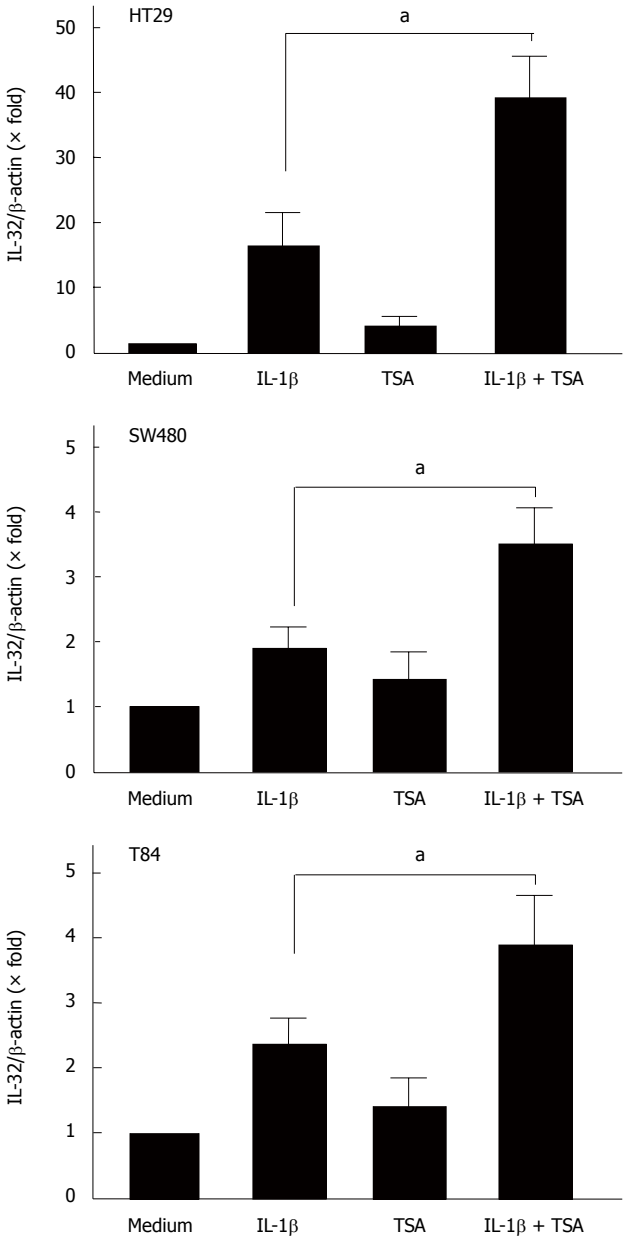


Figure 5 Effects of trichostatin-A (TSA) on IL-32α mRNA expression. The cells (HT-29, SW480, T84) were incubated for 12 h with IL-1β (50 ng/mL) in the presence or absence of TSA (5 μmol/L), and then the IL-32α mRNA expression was determined by real-time PCR. The data are expressed as mean ± SD (n = 4). $^{a}P < 0.05$.

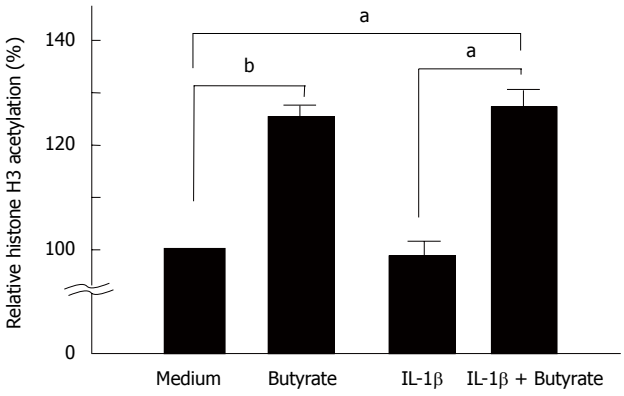


Figure 6 Effects of butyrate on histone H3 acetylation. HT-29 cells were stimulated for 12 h with IL-1β (50 ng/mL) in the presence or absence of butyrate (10 mmol/L). Histone H3 acetylation was then detected by histone H3 acetylation assay kits (Epigentek; Brooklyn, NY, USA). Histone H3 acetylation was expressed as a value relative to medium alone. The data are expressed as mean ± SD (n = 4). $^{a}P < 0.05$, $^{b}P < 0.01$.

DISCUSSION

Among SCFAs, the biological actions of butyrate have been well investigated. Butyrate is easily absorbed in the large intestine, and plays a role as the energy source for intestinal epithelial cells[13]. On the other hand, previous studies have demonstrated that butyrate blocks NF-κB activation in intestinal epithelial cells, and is thought to have an anti-inflammatory effect[25,26]. Based on this hypothesis, some clinical trials have been performed to test the therapeutic effects of butyrate enemas on ulcerative colitis lesions[27-29].

In this study, we examined the effects of SCFAs on IL-32α expression in intestinal epithelial cells. Initially, we hypothesized that SCFAs, in particular butyrate, might suppress IL-32α expression in these cells, since IL-32α expression has been reported to depend on NF-κB activation, and butyrate has been reported to suppress NF-κB activation. However, contrary to our initial expectation, butyrate stimulated IL-32α expression in these cells. This was characterized by two actions: butyrate by itself could stimulate IL-32α expression, and the synergistic effects of butyrate on cytokine-induced IL-32α expression was observed specifically in combination with IL-1β.

Recently, we reported that IL-32 expression mainly depends on the Akt-PI3K signaling pathway and NF-κB activation. As shown in Figure 6, butyrate did not affect Akt phosphorylation, indicating that the Akt-PI3K pathway was not involved in butyrate-mediated IL-32 expression. Furthermore, we have previously shown that butyrate inhibits IL-1β-induced NF-κB activation in HT-29 cells[26,30,31]. This suggests that the modulation of NF-κB activation is not involved in the effects of butyrate on IL-1β-induced IL-32 expression. This concept is supported by the finding that butyrate did not affect IL-32 expression by TNF-α, which is a potent stimulator of NF-κB activation[32,33]. Thus, the mode of action of butyrate on IL-32 expression in intestinal epithelial cells is mediated by different mechanisms from those reported previously.

It is generally accepted that butyrate leads to the development of reversible histone hyperacetylation *via* the inhibition of histone deacetylase activity[14,15]. Reversible histone hyperacetylation is now believed to play an important role in the regulation of chromatin structure[34,35]. Histone hyperacetylation leads to a more relaxed chromatin structure, and thus facilitates transcription factor access to the promoter regions of certain genes without directly initiating gene transcription[23,24]. Based on this knowledge, the mechanisms by which butyrate selectively induces the increase in IL-1β-induced IL-32 gene expression might be explained by an enhancement of transcription by histone hyperacetylation. This was supported by the effects of trichostatin A, a potent histone deacetylase

inhibitor, on IL-32 expression in these cells. On the other hand, although the precise molecular mechanisms remain to be investigated, increased transcriptional activity by histone hyperacetylation might not account for the effects of TNF-α- and IFN-γ-on IL-32 expression. In order to clarify the precise mechanisms by which butyrate specifically stimulates IL-1β-stimulated IL-32 expression, further studies such as the effects of butyrate on signaling pathways to induce IL-32 expression are needed.

Clinical application of this study is limited because the role of IL-32α in inflammatory bowel disease (IBD) is still obscure. Netea *et al*[2] demonstrated recently that IL-32 augments the production of IL-1α, TNF-α, IL-6 and IL-8 by means of the nucleotide-binding oligomerization domain proteins (NOD1 and NOD2) through a caspase-1-dependent mechanism. NODs are a family of intracytoplasmic bacterial peptidoglycans which subsequently induce NF-κB activation. Mutations in *NOD2* have been implicated in the pathogenesis of Crohn's disease (CD). Recently, it has been shown that the *NOD2* mutation in CD patients potentiates NF-κB activity and IL-1α processing. Thus, these findings suggest a pivotal role of IL-32 in the pathophysiology of IBD, and CD in particular. Our study also augments the proinflammatory aspect of butyrate.

In conclusion, we observed that butyrate stimulates IL-32α expression in intestinal epithelial cells. Since IL-32α is considered to be a proinflammatory cytokine, our finding might be a part of the proinflammatory picture of butyrate in mucosal inflammatory responses in the intestine. As the next step, the *in vivo* effects of butyrate on mucosal IL-32 expression should be investigated in the future.

COMMENTS

Background

Recently, interleukin (IL)-32 was defined as a proinflammatory cytokine characterized by the stimulation of secretion of IL-1β, tumor necrosis factor (TNF)-α, IL-6, and IL-8. Dietary fiber (nonstarch polysaccharides) and resistant starch escape digestion in the upper gastrointestinal tract, and undergo anaerobic bacterial fermentation in the colon. This process produces short-chain fatty acids (SCFAs), predominantly acetate, propionate, and butyrate, as the major by-products.

Research frontiers

The authors have previously reported that IL-32α is overexpressed in inflammatory bowel disease (IBD). IL-32α was overexpressed in the inflamed mucosa of IBD patients and was strongly induced by IL-1β, IFN-γ and TNF-α. However, the role of IL-32α is yet to be elucidated. In this study, they investigated the *in vitro* effects of butyrate on IL-32α expression in human intestinal epithelial cell lines.

Innovations and breakthroughs

The authors observed that butyrate stimulates IL-32α expression in intestinal epithelial cells. Since IL-32α is considered to be a proinflammatory cytokine, their finding might be a part of the proinflammatory picture of butyrate in mucosal inflammatory responses in the intestine.

Applications

Clinical application of this study is limited because the role of IL-32α in IBD is still obscure. A recent report demonstrated that IL-32 augments the production of IL-1α, TNF-α, IL-6 and IL-8 by means of the nucleotide-binding oligomerization domain proteins (NOD1 and NOD2) through a caspase-1-dependent mechanism. NODs are a family of intracytoplasmic bacterial peptidoglycans which subsequently induce NF-κB activation. Mutations in *NOD2* have been implicated in the pathogenesis of Crohn's disease (CD). Recently, it has been shown that the *NOD2* mutation in CD patients potentiates NF-κB activity and IL-1α processing. Thus, these findings suggest a pivotal role of IL-32 in the pathophysiology of IBD, and CD in particular. This study also augments the proinflammatory aspect of butyrate.

Terminology

IL-32 was originally reported as natural killer (NK) transcript 4, and has been described as a cytokine produced mainly by T-lymphocytes, NK cells, epithelial cells, and blood monocytes. The gene encoding IL-32 is located on human chromosome 16p13.3 and is organized into eight exons. There are four splice variants, and IL-32α is reported as the most abundant transcript. Recently, IL-32 was defined as a proinflammatory cytokine characterized by the stimulation of secretion of IL-1β, TNF-α, IL-6, and IL-8 *via* the activation of p38 mitogen-activated protein kinases, nuclear factor (NF)-κB and activating protein-1 signal transduction pathways. Recently, the authors reported the overexpression of IL-32α in the inflamed mucosa of IBD. *In vitro* experiments on human intestinal epithelial cell lines showed that IL-32α expression was induced by IL-1β, IFN-γ and TNF-α through the activation of Akt-phosphatidylinositol 3-kinase.

Peer review

Kobori *et al* present an interesting study of a novel pro-inflammatory cytokine IL-32. They investigated the effects of butyrate on IL-32α expression in epithelial cell lines.

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ORIGINAL ARTICLE

Dicoumarol enhances gemcitabine-induced cytotoxicity in high NQO1-expressing cholangiocarcinoma cells

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Abstract

AIM: To investigate whether dicoumarol, a potent inhibitor of NAD(P)H quinone oxidoreductase-1 (NQO1), potentiates gemcitabine to induce cytotoxicity in cholangiocarcinoma cells (CCA) and the role of reactive oxygen generation in sensitizing the cells.

METHODS: Four human cell lines with different NQO1 activity were used; the human CCA cell lines, KKU-100, KKU-OCA17, KKU-M214, and Chang liver cells. NQO1 activity and mRNA expression were determined. The cells were pretreated with dicoumarol at relevant concentrations before treatment with gemcitabine. Cytotoxicity was determined by staining with fluorescent dyes. Oxidant formation was examined by assay of cellular glutathione levels and reactive oxygen species production by using dihydrofluorescein diacetate. Measurement of mitochondrial transmembrane potential was performed by using JC-1 fluorescent probe. Western blotting analysis was performed to determine levels of survival related proteins.

RESULTS: Dicoumarol markedly enhanced the cytotoxicity of gemcitabine in KKU-100 and KKU-OCA17, the high NQO1 activity and mRNA expressing cells, but not in the other cells with low NQO1 activity. Dicoumarol induced a marked decrease in cellular redox of glutathione in KKU-100 cells, in contrast to KKU-M214 cells. Dicoumarol at concentrations that inhibited NQO1 activity did not alter mitochondrial transmembrane potential and production of reactive oxygen species. Gemcitabine alone induced activation of NF-κB and Bcl-$_{xL}$ protein expression. However, gemcitabine and dicoumarol combination induced increased p53 and decreased Bcl-$_{xL}$ levels in KKU-100, but not in KKU-M214 cells.

CONCLUSION: NQO1 may be important in sensitizing cells to anticancer drugs and inhibition of NQO1 may be a strategy for the treatment of CCA.

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Key words: NAD(P)H quinone oxidoreductase-1; Dicoumarol; Cholangiocarcinoma; Chemotherapy; Oxidative stress

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INTRODUCTION

NAD(P)H quinone oxidoreductase-1 (NQO1 or DT-diaphorase) is a ubiquitous flavoprotein localized widely in body tissues. It is an obligated two-electron reductase that reduces quinones to hydroquinones, thus bypassing the toxic semiquinone intermediates, and these resultant hydroquinones are thus ready for further conjugation and excretion[1]. Several functions of NQO1 have been proposed including xenobiotic detoxification, superoxide scavenging, maintenance of endogenous antioxidant, modulation of p53 and proteasomal degradation[2-4]. It is conceivable that NQO1 functions primarily to protect normal cells from oxidant stress and electrophilic attack. A number of experimental models and epidemiological studies support the concept that intake of dietary phytochemicals confers a cancer chemoprevention effect and these chemicals have been shown to induce increased expression of phase Ⅱ drug detoxifying enzymes including NQO1[5,6]. The cytoprotective role of NQO1 is supported by reports that disruption of the *NQO1* gene or genetic polymorphism increase the risk of chemical-induced toxicity and carcinogenesis[7,8]. The expressions of *NQO1* and antioxidant enzymes are recognized as an adaptive response to chemical stress[6,9]. On the other hand, analysis of several solid tumors found an over-expression of the *NQO1* gene in cancers of the liver, thyroid, breast, colon, lung, and pancreas[10,11]. Under these circumstances, NQO1 probably functions to protect cancer cells by eliminating oxidant species and making cells resistant to anticancer drugs that induce oxidative injury[12].

Inhibition of NQO1 activity by dicoumarol has been shown to suppress urogenital and pancreatic cancer cell growth and potentiate cytotoxicity of cisplatin and doxorubicin[13,14]. The inhibition of NQO1 with dicoumarol was suggested to stimulate formation of superoxide, oxidative stress and subsequent suppression of pancreatic cancer cell growth and induction of apoptosis[15,16]. However, dicoumarol has been shown to induce formation of reactive oxygen species (ROS) independently from NQO1 activity by inhibition of the mitochondrial electron transport chain[17]. Therefore, the question as to whether inhibition of NQO1 renders cancer cells more sensitive to chemotherapeutic agents is still not clear. More study is necessary to define the role of NQO1 in cancer cells. Sensitizing cancer cells to be more susceptible to radiotherapy or chemotherapy may be an important strategy to overcome resistance in cancer chemotherapy.

Cholangiocarcinomas (CCA) are rare types of liver cancers arising from the biliary duct system. Surgical resection with a histologically free margin is the only chance for cure[18]. Unfortunately, only few patients are eligible for surgery. Furthermore, current chemotherapy and radiotherapy regimens do not substantially improve survival in CCA patients[18,19]. Gemcitabine has been the most important nucleoside analog which has wide spectrum activity against various solid and hematological tumors[20]. It shows some efficacy and is well tolerated in CCA patients[21]. It is essential to gain insight into molecular mechanisms by which cancer cells operate to survive and evade the attack by chemotherapeutic agents.

Because NQO1 appears to be a potential target for exploitation in cancer chemotherapy, we investigated whether dicoumarol, a potent inhibitor of NQO1, sensitized CCA cells to respond to the cytotoxicity of gemcitabine. Furthermore, we examined whether dicoumarol- and gemcitabine-induced cell killing was associated with ROS generation, mitochondrial dysfunction and apoptotic protein expression.

MATERIALS AND METHODS

Human cell line cultures

Three human CCA cell lines established in our institute, KKU-100, KKU-OCA17 and KKU-M214, were derived from human intrahepatic CCA tissues with the histological types of poorly differentiated, well differentiated and moderately differentiated adenocarcinoma, respectively[22,23]. Chang liver cells were also used in the study. CCA cells and Chang liver cells were routinely cultured as previously described[24] in Ham's F12 media, supplemented with 4 mmol/L L-glutamine, 12.5 mmol/L N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), at pH 7.3, 100 U/mL penicillin 100 μg/mL streptomycin sulfate and 10% fetal calf serum. The media was renewed every 3 d, trypsinized with 0.25% trypsin-EDTA and subcultured in the same media.

NQO1 activity assay

NQO1 assay was performed essentially according to a previously published method[25]. Cells were seeded onto 96-well cultured plates overnight. Cells were then lysed with 50 μL of 0.8% digitonin in 2 mmol/L EDTA at room temperature for 10 min. The assay was performed using menadione and MTT [3-(4,5-dmethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole] in the substrate coupling reaction and measured as rate-kinetics in a micro-plate reader at a wavelength of 620 nm. The initial velocity of reaction was calculated as NQO1 activity using the extinction coefficient of formazan of MTT of 11 300 L/mol per cm and correction factor for the light path. In the determination of the enzymatic property of NQO1 in each cell line, cells were incubated with varying concentrations of dicoumarol for 10 min before carrying out assays as above.

Cytotoxicity assay

CCA cells (KKU-100, KKU-OCA17, and KKU-M214) and Chang liver cells were seeded onto 96-well cultured plates at a density of 5×10^3 cells/well (KKU-M214 and Chang cells) or 7.5×10^3 cells/well (KKU-100 and KKU-OCA17) for an overnight, then media was renewed with fresh media containing test compound and further incubated for the indicated times. The cytotoxicity was determined by fluorescence microscopy[26]. In brief, cells were washed once with phosphate-buffered saline (PBS) and the following added: 4 μL mixture of acridine orange, ethidium bromide (each 1 μg/mL) and trace amount of he-

moglobin. The cells were examined using a Nikon Eclipse TS100 inverted microscope with excitation and emission filters of 480 and 535 nm, respectively. The microphotographs were taken at predetermined three areas per well in triplicate wells per concentration with a Nikon Coolpix digital camera. The numbers of viable and non-viable cells were counted. The viable cells were colored green with intact nuclei. The non-viable cells included necrotic and apoptotic cells which showed bright orange staining and green fluorescence, with appearance of cell shrinkage and condensation and fragmentation of the nuclei, respectively. The antiproliferation value was calculated as = (number of viable cells in control wells - viable cells in treatment wells)/(number of viable cells in control wells) × 100.

Determinations of glutathione and glutathione disulfide

Total glutathione assay was performed essentially according to Tietze methods[27]. Glutathione disulfide (GSSG) was assayed by the previously described method[28] using 1-methyl-2-vinylpyridinium triflate (M2VP) as a glutathione scavenger. Cell cultures were trypsinized and washed three times with cold PBS buffer and centrifuged at 1500 *g*, 4℃ for 10 min and resuspended in PBS buffer. Cell suspension of 100 μL was reacted with M2VP (33 mmol/L in DI) or without M2VP. The solution was mixed gently and stored frozen at -20℃ until analysis. An aliquot of cell suspension was saved for protein determination by Bradford's dye binding assay.

Measurement of mitochondrial transmembrane potential

To measure the change in mitochondrial transmembrane potential ($\Delta\Psi_m$), the lipophilic cation fluorescent dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolylcarbocyanine iodide) was used. After treatment with dicoumarol, gemcitabine or the combination at defined period of times, cultured cells were loaded with JC-1 mitochondrial membrane potential assay kit (Clayman Chemical, Ann Arbor, Michigan) by incubation for 30 min at 37℃. After that, cultured cells were rinsed, incubated in JC-1 assay buffer and mitochondrial transmembrane potential was analyzed by a fluorescent plate reader. In healthy mitochondria, JC-1 forms J-aggregates which display strong fluorescent intensity with excitation and emission wavelength at 560 and 595 nm, respectively. In depolarized mitochondria, JC-1 exists as J monomers which show strong fluorescence with excitation and emission wavelength at 485 and 535 nm, respectively. The shift down in ratio of fluorescent intensity of JC-1 aggregates to fluorescent intensity of monomers is used as an indicator of depolarization of $\Delta\Psi_m$.

Determination of formation of reactive oxygen species

Reactive oxygen species (ROS) levels generated from cultured cells were determined by incubating the cells in Hank's buffer supplemented with 15 mmol/L HEPES containing 1.2 μg/mL dihydrofluorescein diacetate (H_2-DHFDA) (Sigma-Aldrich, St. Louis, MO) for 30 min at 37℃. H_2-DHFDA was taken up into the cells, hydrolyzed and oxidized to the fluorescent product DHF by ROS. The fluorescent signal was determined by a fluorescent plate reader with a setting of the excitation and emission wavelengths at 485 and 520 nm, respectively.

RNA isolation and reverse transcription-polymerase chain reaction

Total RNA was extracted from liver tissues and the four cell lines using Trizol®LS reagent following the manufacturer's instructions. Total RNA (3 μg) was reverse-transcribed in 20 μL containing 0.5 μg of oligo(dT)$_{15}$ primer, 20 U of RNasin® ribonuclease inhibitor and 200 U of ImProm-Ⅱ™ reverse transcriptase in 10 × PCR buffer, 3 mmol/L $MgCl_2$, and 1 mmol/L dNTPs. The first-strand cDNA was synthesized at conditions of 42℃ for 60 min. The reverse transcription products served as a template for real-time PCR. PCR amplification was performed using specific primers for the NQO1 and the internal control using FDFT1. The PCR primer sequences were as follows: NQO1; forward primers: 5'GGCAGAAGAGCACTGATCGTA3', NQO1; reverse primers: 5'TGATGGGATTGAAGTTCATGGC3', GenBank accession number BC007659.2, FDFT1; forward primers: 5'TTTAACTTCTGTGCTATTCCAC3', FDFT1; reverse primers: 5'TCTCCAGTCTGAACATAGTC3', GenBank accession number NM_004462.3. The real-time fluorescence PCR, based on SYBR Green, was carried out in a final volume of 20 μL containing 1 × SYBR Green PCR Master Mix (DyNAmo™ Flash SYBR® Green qPCR Kit), 0.5 μmol/L of each NQO1 or FDFT1 primer. Thermal cycling was performed for each gene in duplicate on cDNA samples in 96-well reaction plates using the ABI 7500 Sequence Detection system (Applied Biosystems). A negative control was included in the experimental runs. The negative control was set up by substituting the template with deionized H_2O and this routinely had a high Ct value which represented the lower detection limit. Real-time PCR was conducted with the following cycling conditions: 95℃ for 10 min, followed by 40 cycles of 95℃ for 15 s, 55℃ for 30 s and 72℃ for 45 s. To verify the purity of the products, a melting curve analysis was produced after each run. Upon completion of 40 PCR amplification cycles, there was a dissociation step of ramping temperature from 60℃ to 95℃ steadily for 20 min, while the fluorescence signal was continually monitored, for melting curve analysis. The relative expression ratio (R) of target genes is calculated based on efficiency (E) and Ct deviation and expressed in comparison to a reference gene. The corresponding real time PCR efficiencies were calculated according to the equation $E = 10^{[-1/slope]}$. All data were analyzed using Sequence Detector Software Version 1.4 (Applied Biosystems).

Western blotting analysis of whole cell and nuclear protein extracts

The whole cell lysates and nuclear protein were prepared according to a previous report[29]. Treated KKU-100 and KKU-M214 cells were washed with PBS, collected and lysed at 4℃ with cell lysis buffer [20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L Na_2EDTA, 1 mmol/L EGTA, 1% Triton, 2.5 mmol/L sodium py-

rophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na_3VO_4, 1 μg/mL leupeptin, 1 mmol/L dithiothreitol, 0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF)] with vigorous shaking. Following centrifugation at 10000 *g* for 15 min, supernatant was collected and stored at -80℃ until use. Nuclear protein was prepared by lysis of cultured cells with hypotonic buffer A [10 mmol/L HEPES-KOH pH 7.9, 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, 0.5 mmol/L DTT, 0.2 mmol/L PMSF, 0.1 mmol/L EGTA], incubated in an ice bath for 15 min and then 1% NP-40 was added, cells were centrifuged at 12000 *g*, 4℃ for 15 min, the nuclear pellet was resuspended in ice-cold buffer B (20 mmol/L HEPES-KOH pH 7.9, 25% glycerol, 1.5 mmol/L $MgCl_2$, 420 mmol/L NaCl, 0.2 mmol/L EDTA, 0.5 mmol/L DTT, 0.2 mmol/L PMSF, and 1 mmol/L EGTA), followed by incubation at 0℃ for 45 min. After vortex mixing, the suspension was centrifuged at 12000 *g*, 4℃ for 30 min. The supernatant containing nuclear proteins was stored at -80℃ for the NF-κB Western immunoblot analysis.

The protein samples were mixed with 5 × loading dye buffer, heated at 95℃ for 5 min and proteins were separated by electrophoresis in 10% SDS-polyacrylamide gel. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes at 50 V for 2 h. The PVDF membranes were blocked for 1 h at room temperature with 5% (w/v) skimmed milk powder in Tris buffer saline (TBS) with 0.1% Tween-20. PVDF membrane was incubated overnight at 4℃ with primary antibodies of rabbit polyclonal IgG NF-κB p65 subunits (dilution 1:500) (sc-109: Santa Cruz Biotechnology), mouse monoclonal IgG Bcl-xL (1:1000) (sc-8392), rabbit polyclonal IgG Bax (1:2000) (sc-493), rabbit polyclonal IgG cyclin D1 (1:1500) (sc-718), and mouse monoclonal IgG1 β-actin (1:2500) (sc-8432) diluted with TBS. The primary antibody was then removed and the blots were extensively washed with TBS/Tween-20. Blots were then incubated for 2 h at room temperature with the secondary antibody horseradish peroxidase goat anti-mouse IgG (sc-2005) and goat anti-rabbit IgG (sc-2004) at 1:5000 dilutions in TBS buffer. After removal of the secondary antibody and washes in TBS buffer, the blots were incubated in the ECL substrate solution (Supersignal® West Pico Chemiluminescent Substrate). Densities of the specific bands of NF-κB, Bcl-xL, Bax, cyclin D1, and β-actin were visualized and captured by Imagequant 350 (GE Healthcare).

Statistical analysis

Data are expressed as mean ± SE of duplicate assays from three independent experiments. An analysis of variance with repeated measurement was used to determine significant differences between each experimental group. The level of significance was set at $P < 0.05$.

RESULTS

Activity and expression of NQO1 in CCA cells

Three CCA cell lines with different histological backgrounds were employed in this study for assessing the status of NQO1. The CCA cells illustrated varying degrees of NQO1 activity and mRNA expression. KKU-100 and KKU-OCA17 cells had high NQO1 activity, whereas KKU-M214 showed low NQO1 activity. Moreover, Chang liver cells, which were derived from normal liver tissue, showed relatively low NQO1 activity and were comparable to KKU-M214 cells (Figure 1A). Consistently, NQO1 mRNA expression in KKU-100 cells showed higher levels than in the other cell lines (Figure 1B).

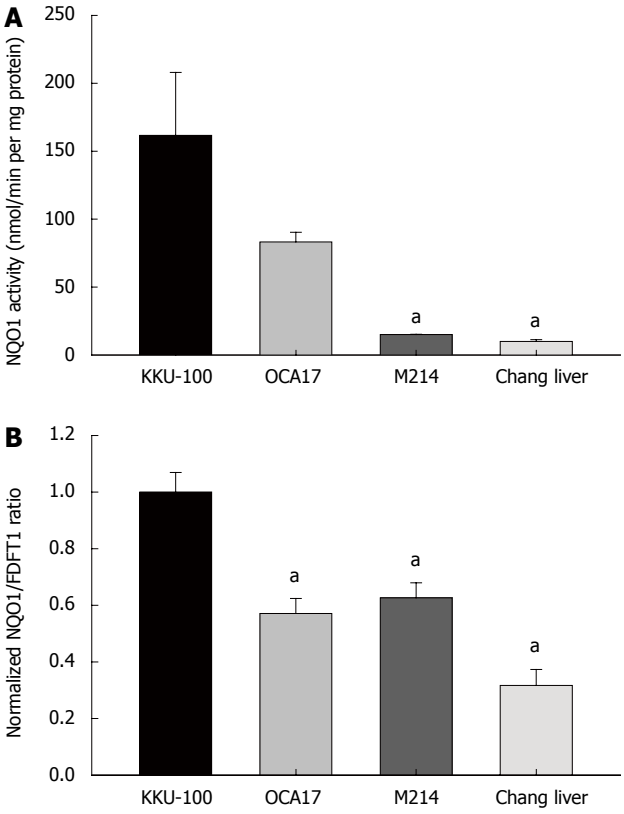


Figure 1 NQO1 activity and mRNA expression of cultured cells. A: NQO1 activity: Cholangiocarcinoma cells, KKU-100, KKU-OCA17, KKU-M214, and Chang liver cells were cultured in 96-well plates for assay of NQO1 activity by enzymatic methods; B: Expression of NQO1 mRNA: The cells were cultured in 6-well plates. Total RNA was extracted by Trizol reagent, converted to cDNA and analyzed by real-time PCR using FDFT1 as internal control. Bars represent mean ± SE, each from 3 experiments. $^aP < 0.05$ *vs* control group.

Sensitivity of NQO1 to dicoumarol

Dicoumarol is a very potent inhibitor of NQO1 activity. All four cell lines were tested against dicoumarol. NQO1 in CCA and Chang cell lysates were assayed for kinetics of inhibition by dicoumarol. The inhibition of NQO1 activity was rapid, within 10 min, and was apparent with a comparable potency among the 4 cell lines (Figure 2) with IC50 of 0.15 ± 0.07, 0.15 ± 0.07, 0.24 ± 0.14 and 0.10 ± 0.22 μmol/L for KKU-100, KKU-OCA17, KKU-M214 and Chang cells, respectively. Since the enzyme activity in all cell lines was almost completely abolished at a dicoumarol concentration of 10 μmol/L, in subsequent experiments dicoumarol was used at the concentration of 10 μmol/L.

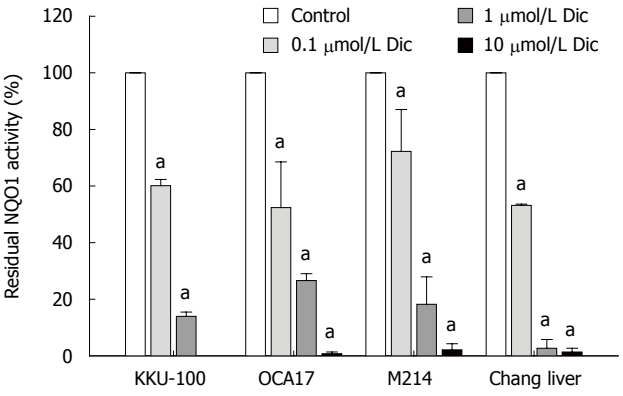


Figure 2 Concentration response of NQO1 inhibition by dicoumarol. KKU-100, KKU-OCA17, KKU-M214, and Chang liver cells were cultured in 96-well plates overnight. The NQO1 activity was assayed in the presence of the inhibitor dicoumarol at concentrations from 0.1-10 μmol/L. Bars represent mean ± SE, each from 3 experiments. $^aP < 0.05$ *vs* control group.

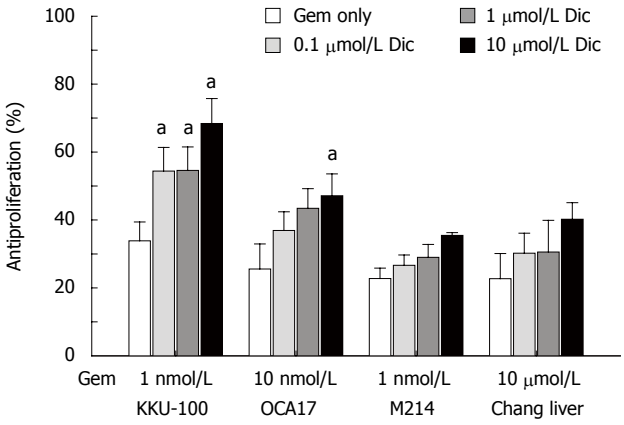


Figure 3 Potentiation of cytotoxicity of gemcitabine by dicoumarol. KKU-100, KKU-OCA17, KKU-M214, and Chang liver cells were cultured in 96-well plates overnight. Cultured cells were pretreated with dicoumarol (0.1-10 μmol/L) for 4 h before treatment with gemcitabine at 1 nmol/L, 10 nmol/L, 1 nmol/L and 10 μmol/L, for KKU-100, KKU-OCA17, KKU-M214, and Chang liver cells, respectively. Antiproliferation was analyzed by staining the cells with fluorescent dyes before examination under fluorescent microscope. Bars represent mean ± SE, each from 3 experiments. $^aP < 0.05$ *vs* control group.

Enhancement of cytotoxicity of gemcitabine by dicoumarol

To investigate whether inhibition of cellular NQO1 activity was associated with increased anticancer drug sensitivity, CCA cells were preincubated with dicoumarol at varied concentrations for 4 h followed by an addition of gemcitabine at predetermined concentrations. The concentrations of gemcitabine that caused cytotoxicity of about 20%-30% were used in the experiments. Those for KKU-100, KKU-OCA17, KKU-M214 and Chang cells were 1, 10, 1 nmol/L and 10 μmol/L, respectively. Treatment with dicoumarol alone at concentrations of 0.1-10 μmol/L caused modest cytotoxicity after incubation for 24 h. Dicoumarol at a concentration of 10 μmol/L induced ⩽ 10% cytotoxicity in all cell types.

Combination of gemcitabine and dicoumarol produced a markedly enhanced cytotoxic effect, particularly in KKU-100 and KKU-OCA17 cells. The enhanced cytotoxic effect in both cell lines was conceivably more than a simple additive effect of the drug and dicoumarol (Figure 3). The cytotoxicity of gemcitabine in KKU-100 cells was enhanced from 34% to 68% and in KKU-OCA17 cells from 25% to 47% in the presence of dicoumarol. On the other hand, the drug combination produced only an additive cytotoxicity in KKU-M214 and Chang cells, i.e. increased from 23% to 36% and 23% to 40%, respectively. It is noted that KUU-100 and KKU-OCA17 cells are high NQO1 activity cells when compared with KKU-M214 and Chang cells.

Dicoumarol-induced oxidative stress in CCA cells

The interactive effect of dicoumarol with gemcitabine in enhancing cytotoxicity was explored with regard to whether dicoumarol induced cellular stress, rendering the cells more susceptible to gemcitabine. In this study, KKU-100 and KKU-M214 cells were employed as representatives of high and low NQO1 activity cells, respectively. The basal total GSH levels in KKU-M214 were higher than that in KKU-100 cells and significantly increased by the treatment with dicoumarol (10 μmol/L) (Figure 4A). On the other hand, glutathione disulfide (GSSG) levels in KKU-100 were dramatically increased with the treatment of dicoumarol, whereas the levels in KKU-M214 were decreased (Figure 4B). This indicates that the two cell types have different oxidative responses after the treatment with dicoumarol.

Determination of the mitochondrial transmembrane potential and formation of reactive oxygen species

Disruption of $\Delta\Psi_m$ is recognized as one of the critical steps leading to apoptotic cell death. The integrity of the inner mitochondrial membrane can be assessed by monitoring of the potential gradient across the membrane using the fluorescent dye JC-1. There was no change in $\Delta\Psi_m$ in cells after treatment with dicoumarol, gemcitabine or the drug combination in both cell types, at an early time (6 h) as well as at 24 h (Figure 5A and B). In contrast, dicoumarol at excessive concentrations (50 and 150 μmol/L) showed a rapidly depolarized $\Delta\Psi_m$, as shown by decrease in the ratio of fluorescent intensity of JC-1 aggregates/JC-1 monomers after 3 h incubation (Figure 5C). The formation of ROS by cultured cells was monitored by a fluorescent dye, DHFDA. It was apparent that there was no increase in ROS formation in cells treated with dicoumarol, gemcitabine or the combination in both cell lines (data not shown), even at a concentration and time where cell apoptosis was evident. High concentrations of dicoumarol also did not induce ROS production, even at those concentrations which caused cell death.

Alteration of survival response proteins induced by gemcitabine and dicoumarol

In order to understand how the combination of gemcitabine and dicoumarol enhanced cytotoxicity in high NQO1 activity cells, proteins related to cell survival were analyzed by Western immunoblotting. Protein p53 is a tumor suppressor protein that responds to various noxious stimuli.

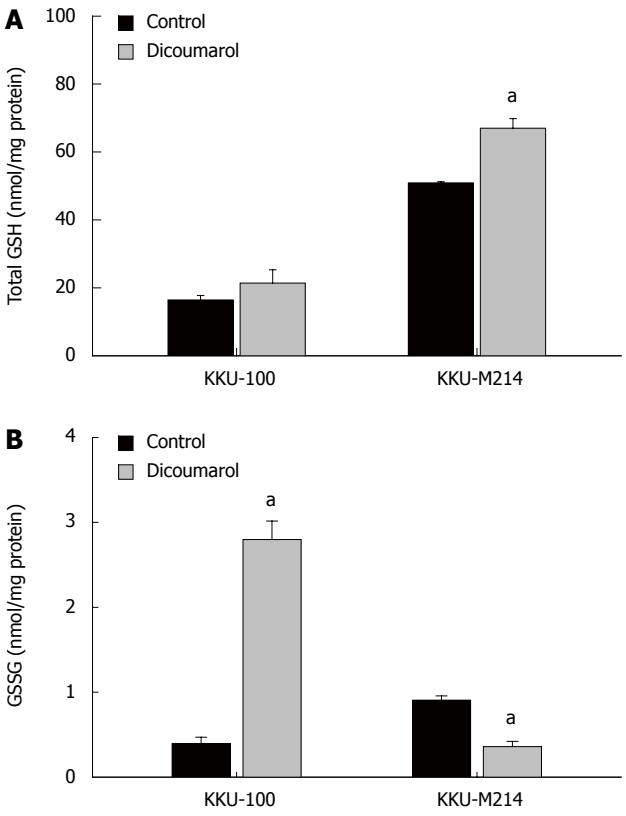


Figure 4 Glutathione redox status in cells treated with dicoumarol. KKU-100 and KKU-M214 cells were cultured and treated with dicoumarol (10 μmol/L) for 4 h, and cells were scraped for assays of (A) total glutathione and (B) glutathione disulfide. Bars represent mean ± SE, each from 3 experiments. $^{a}P < 0.05$ *vs* control group.

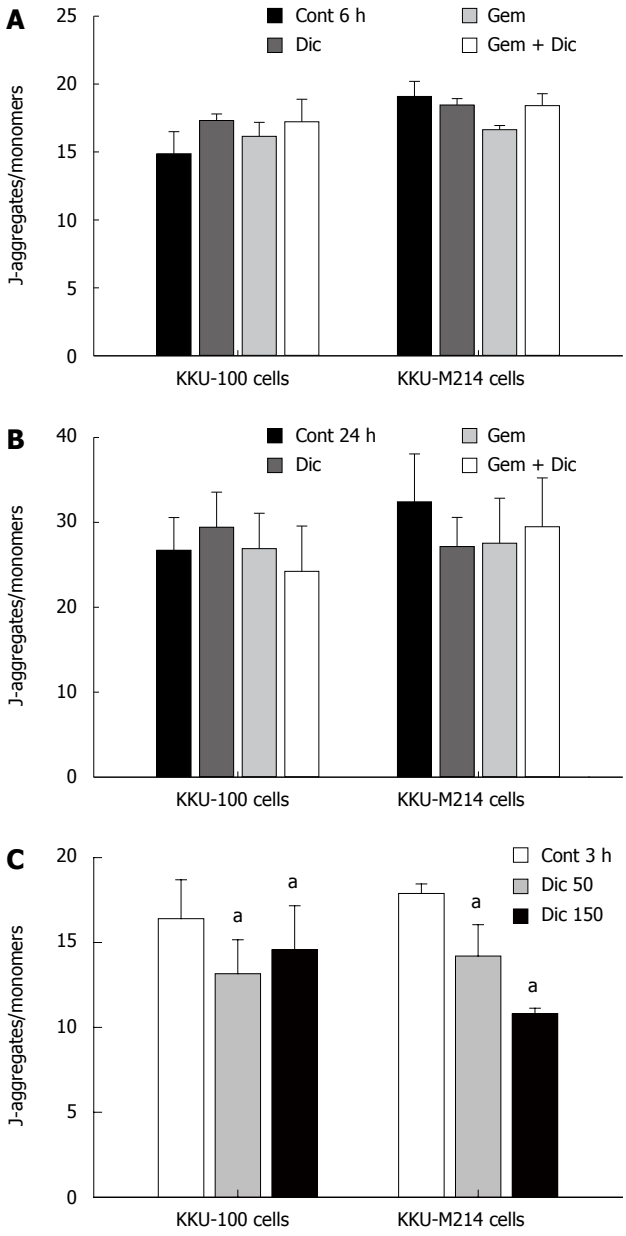


Figure 5 Assay of mitochondrial transmembrane potential and reactive oxygen in CCA cells. The mitochondrial transmembrane potential was analyzed by using JC-1 fluorescent probe. Fluorescent readings of the J-aggregates and J monomers were used as measurement of mitochondrial transmembrane potential. KKU-100 and KKU-M214 cells were cultured in 96-well black plates. The cultured cells were pretreated with dicoumarol at 10 μmol/L for 4 h, then gemcitabine at 1 nmol/L was added and incubated at various times. A: Incubation for 6 h; B: Incubation for 24 h; C: Other cultured cells were treated with dicoumarol at 50 and 150 μmol/L for 3 h. Bars represent mean ± SE, each from triplicate assay. $^{a}P < 0.05$ *vs* control group.

Dicoumarol or gemcitabine treatment induced increased p53 levels in KKU-100 cells and the level was further increased during the treatment with the drug combination. On the other hand, dicoumarol or gemcitabine or the combination did not alter p53 levels in KKU-M214 cells. Bcl-2 proteins consist of proapoptotic (Bax) and antiapoptotic proteins (Bcl-xL) where they regulate mitochondria outer membrane permeabilization. Bcl-xL levels were induced by gemcitabine in both cell types, however, the combination of gemcitabine with dicoumarol suppressed elevated Bcl-xL levels in KKU-100 cells but not in KKU-M214 cells. On the other hand, the changes in Bax levels were found with a similar pattern in both cell types, i.e. Bax levels were induced with gemcitabine, while drug combination induced no changes when compared with controls. Cyclin D1 levels were decreased upon the treatment with dicoumarol in both cell types, but not by gemcitabine. However, the drug combination apparently suppressed cyclin D1 levels when compared with gemcitabine alone.

The expression of the nuclear p65 subunit of NF-κB was slightly increased after the treatment with gemcitabine and showed little change induced by the co-treatment with dicoumarol in both cell lines. Figure 6 shown the Western analysis of proteins related to survival.

DISCUSSION

NQO1 is one of the attractive targets in development of chemotherapy, since NQO1 may function to protect normal cells, as well as tumor cells, particularly when it is highly expressed[12,30]. Suppression of NQO1 induced by proinflammatory cytokines or genetic defects may render normal cells susceptible to oxidant-mediated cell toxicity and carcinogenesis[7,8,22]. Inhibition of NQO1 activity with dicoumarol at relevant concentrations which produced modest cytotoxicity when administered alone potentiated gemcitabine-induced cytotoxicity in cells with high NQO1 activity. The combination of dicoumarol with gemcitabine

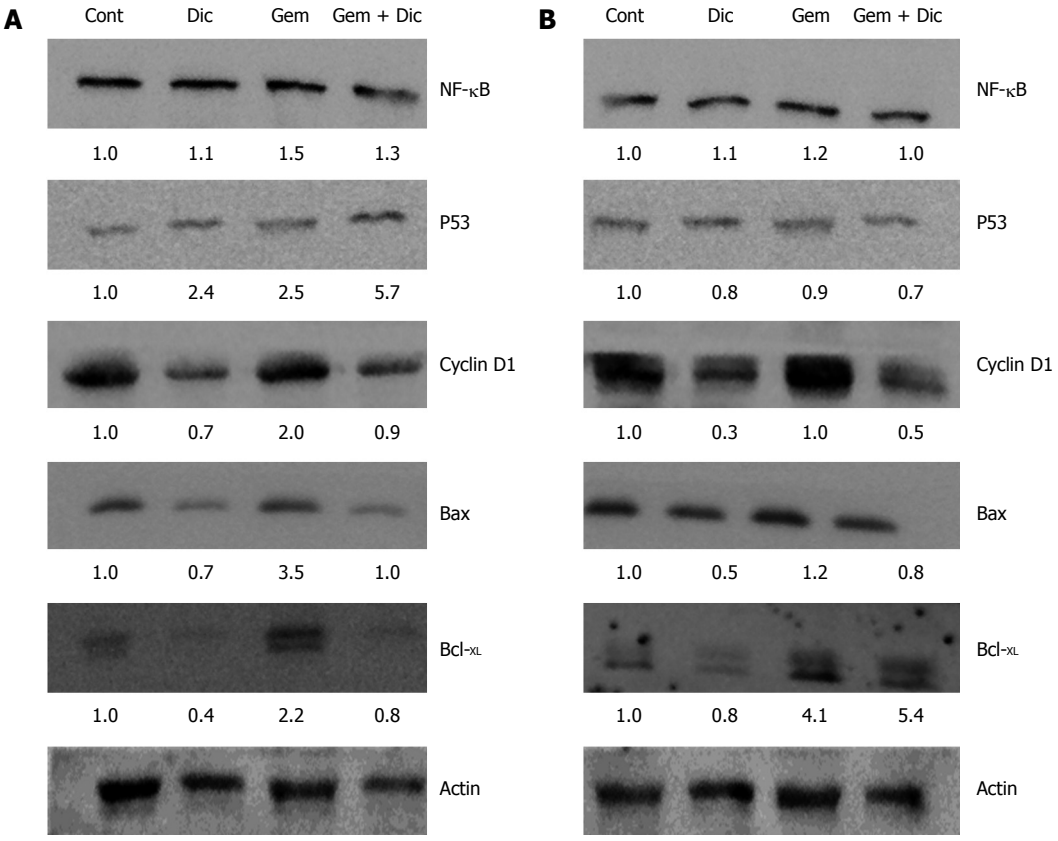


Figure 6 Western blotting analysis of proteins related to survival. KKU-100 and KKU-M214 cells were cultured overnight, pretreated with 10 μmol/L dicoumarol for 4 h before being treated with 1 nmol/L gemcitabine for 24 h. Cultured cells were collected for Western blotting analysis using β-actin as an internal control for equal protein loading. A: KKU-100 cells; B: KKU-M214 cells. Cont: Controls; Dic: Dicoumarol alone; Gem: Gemcitabine alone; Gem + Dic: Combination of gemcitabine and dicoumarol. Values were an average from two experiments of the target protein normalized with the internal control.

enhanced p53 and decreased Bcl-xL expression, and this may be related to the mechanism of cell sensitization and killing.

NQO1 is well known to function as a drug metabolizing and antioxidant enzyme, protecting the cell from oxidative injury[3]. Previous studies suggested that NQO1 modulates p53 expression by interference with 20S proteasome-mediated degradation of p53[3,31]. *P53* is a tumor suppressor gene that upon stimulation by DNA damage or oxidative stress, induces either growth arrest or apoptosis[32]. Our study showed that dicoumarol increased levels of p53 in KKU-100 cells, but these levels were unchanged in KKU-M214 cells. The increased p53 expression, together with decreased Bcl-xL protein expression, was associated with enhanced cytotoxicity of gemcitabine. However, dicoumarol has been previously reported to decrease p53 protein levels and this was associated with either inhibition or induction of apoptosis in myeloid leukemic cells or urogenital cancer cells, respectively[14,31]. The discrepancy in p53 levels induced by the treatment with dicoumarol is not readily comprehensible, although our study used much lower concentrations of dicoumarol. Moreover, treatment of gemcitabine apparently induced the activation of NF-κB and expression of Bcl-xL. This increase of antiapoptotic proteins may be a survival signal engaged in response to gemcitabine[33]. Interestingly, the combination with dicoumarol diminished the expression of Bcl-xL in KKU-100 cells concurrent with potentiation of the cytotoxicity of gemcitabine, while this did not happen in KKU-M214 cells.

Protein p53 and the Bcl-2 protein family regulate mitochondrial outer membrane permeabilization[34]. Induction of apoptotic cell death is often associated with disruption of the mitochondrial inner membrane which is reflected as dissipation of $\Delta\Psi_m$ and formation of mitochondrial permeability transition pore (MPTP) with subsequent cell death[35]. In the treatment with low concentrations of dicoumarol with or without gemcitabine, $\Delta\Psi_m$ appeared to be well maintained according to the assay of JC-1, despite the drug combination inducing marked CCA cell killing. This suggests no disruption of the mitochondrial inner membrane at the concentrations that potentiate gemcitabine cytotoxicity. Likewise, it was shown that prostaglandin A2 induced human promyelocytic leukemia cell death associated with cytochrome c release, with no change in $\Delta\Psi_m$[36]. However, dicoumarol at the high concentrations caused disruption of $\Delta\Psi_m$ and rapid cell killing which is consistent with many previous reports[14,17].

Dicoumarol has been suggested to trigger cell killing *via* increased formation of ROS[15-17]. We did not observe an increased ROS formation when cells were treated with dicoumarol or dicoumarol with gemcitabine combination. Dicoumarol, even at high concentrations

(150 μmol/L) that rapidly killed both types of CCA cells within 3 h of incubation (data not shown), had no significant effect on ROS formation. This suggests that superoxide formation may not be an important mediator of cell killing by dicoumarol[17]; it is probable that dicoumarol exerts its cell killing mechanism on CCA cells differently from other epithelial cells.

GSH, a tripeptide found abundantly in cells, functions as antioxidant and regulates activities of various redox-sensitive proteins[37]. Although dicoumarol did not increase formation of ROS, it induced oxidant stress in KKU-100 cells, but not in KKU-M214 cells, evidently by increasing and decreasing GSSG levels, respectively. The increased cellular oxidant may be implicated in several signal transduction pathways which eventually lead to enhanced susceptibility of KKU-100 cells. Nonetheless, dicoumarol has been reported to show several different pharmacological activities[15,17,38] and apoptotic cell killing may be dissociated with NQO1 inhibition[39], as some synthetic mechanism-based NQO1 inhibitors have shown lack of association between enzyme inhibition and cytotoxic effect. The discrepancy of the reports may be explicable partly on the grounds that the sensitizing effect of dicoumarol may be dependent on inherent activity of NQO1 in these cell types.

In conclusion, NQO1 may play roles in the sensitivity of CCA cells to gemcitabine. When using dicoumarol at relevant concentrations to inhibit NQO1 activity, dicoumarol enhances gemcitabine cytotoxicity in high NQO1 activity CCA cells. Furthermore, the mechanism of dicoumarol-induced cell killing may not be mediated *via* disruption of mitochondrial function and formation of ROS, but may be related to suppression of the pro-survival response to the chemotherapy. NQO1 is a potential target in the development of chemotherapy for tumors with high enzyme expression.

COMMENTS

Background

NAD(P)H quinone oxidoreductase-1 (NQO1) is a drug metabolizing and antioxidant enzyme which plays important roles in protection of cells against electrophiles and oxidants. Some cancers exhibit high activity of NQO1 which suggests a cytoprotective role in the cancer.

Research frontiers

Inhibition of NQO1 activity may abate the adaptive survival response of cancer cells induced by gemcitabine therapy and render cancer cells more susceptible to the drug.

Innovations and breakthroughs

This report shows that dicoumarol, a potent inhibitor of NQO1 at low concentrations, potentiates cytotoxicity of gemcitabine in cholangiocarcinoma cells which inherently have high NQO1 activity. Disruption of cellular glutathione redox may be associated with modulation of redox-sensitive signaling proteins such as p53 and render enhanced cells susceptible to gemcitabine.

Applications

Inhibition of NQO1 is suggested to be a potential strategy for development in cancer chemotherapy, particularly for cholangiocarcinoma which was used in this study.

Peer review

The methodology and the results are appropriate and support the findings in the literature.

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ORIGINAL ARTICLE

Effect of 5-Aza-2′-deoxycytidine on immune-associated proteins in exosomes from hepatoma

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Abstract

AIM: To study the effect of 5-Aza-2′-deoxycytidine (5-Aza-CdR) on heat shock protein 70 (HSP70), human leucocyte antigen-Ⅰ (HLA-Ⅰ) and NY-ESO-1 proteins in exosomes produced by hepatoma cells, HepG2 and Hep3B.

METHODS: Exosomes derived from HepG2 and Hep3B cells treated with or without 5-aza-CdR were isolated and purified by ultrafiltration centrifugation and sucrose gradient ultracentrifugation. The number of exosomes was counted under electron microscope. Concentration of proteins in exosomes was measured by bicinchoninic acid protein assay. Expression of HSP70, HLA-Ⅰ and NY-ESO-1 proteins in exosomes was detected by Western blotting and immunoelectron microscopy. mRNA expression of *p53* gene was detected by reverse transcription polymerase chain reaction.

RESULTS: The mRNA expression of *p53* gene was increased in both hepatoma cell lines after treatment with 5-Aza-CdR. The number of exosomes and the concentration of total proteins in exosomes were increased significantly after treatment with 5-aza-CdR ($P < 0.05$). After treatment with 5-Aza-CdR, immunoelectron microscopy and Western blotting showed that the HSP70, HLA-Ⅰ and NY-ESO-1 proteins were increased in exosomes produced by both hepatoma cell lines.

CONCLUSION: 5-aza-CdR, an inhibitor of DNA methyltransferase, can increase exosomes produced by hepatoma cells and immune-associated protein component of exosomes, which may be mediated by *p53* gene up-regulation and 5-Aza-CdR demethylation.

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Key words: 5-Aza-2′-deoxycytidine; Exosome; Immunomolecule; Hepatoma cell

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INTRODUCTION

Human hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, with an annual incidence of over half a million. Despite the improvements in surveillance, imaging technology, surgical techniques, and perioperative care, its mortality rate still increases, ac-

counting for 53% of all liver cancer deaths worldwide in China[1]. The most effective treatment modalities available for HCC to date are surgical resection, liver transplantation, and ablative therapy[2,3]. However, they are not indicated for patients who have relapse or are at advanced stage of the disease[4]. Moreover, the most commonly used treatment methods for various cancers, such as radiotherapy and chemotherapy, are often excluded in treatment of HCC due to their intolerable toxicity and insensitivity[5]. It is therefore necessary to develop a novel strategy against the progression and recurrence of HCC.

Exosomes secreted by tumor cells have become a recent hot spot in research of tumor immunology, raising intriguing attention to their immune-stimulating function *in vitro* and tumor model experiments[6,7]. However, acquiring a sufficient number of exosomes with a high quality for more powerful immune-stimulating effects has remained a great challenge for tumor immunotherapy[8-10]. Apart from the p53-dependent pathway, the mechanisms by which tumors secrete exosomes have not been well understood[11].

5-Aza-2'-deoxycytidine (5-Aza-CdR), a DNA methyltransferase inhibitor and a demethylation promoter in CpG regions of many genes, including the *p53* gene, can significantly restore or increase their expression[12,13], including the expression of *p53* by damaging DNA[14]. It has been shown that 5-Aza-CdR can significantly increase the expression of immune molecules necessary for antitumor cellular immunity by demethylating DNA, such as human leucocyte antigen (HLA)-Ⅰ, and HLA-Ⅱ, and significantly enhance the therapeutic effect of anti-tumor immunity *in vitro* and in animal experiments[15-17]. However, few reports are available on the effects of 5-Aza-CdR on the secretion of exosomes and the protein level in exosomes. This study was to explore the effect of 5-Aza-CdR on the secretion of exosomes, tumor-associated antigens and immune molecules in exosomes, and its mechanisms by which hepatocellular carcinoma cell lines secrete exosomes, in an attempt to provide preliminary experimental evidence for 5-Aza-CdR-modified exosomes-based anti-hepatoma immunotherapy.

MATERIALS AND METHODS

Materials

HepG2 cell line was generously provided by Professor You-Yong Lu, Beijing Cancer Institute. Hep3B cell line was purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (CAS).

Drugs and reagents

5-Aza-CdR, heavy water, cane sugar (analytically pure) and protein A colloidal gold (SPA) were purchased from Sigma Company (Santa Clara, CA, USA). FBS and DMEM culture media were purchased from GIBCo Company (Carlsbad, CA, USA). Western blotting reagents used in this study included rabbit anti-human heat shock protein 70 (HSP70) polyclonal antibody from Abcam (Cambridge, UK), mouse anti-human HLA-Ⅰ monoclonal antibody from Chemicon (Los Angeles, CA, USA), mouse anti-human NY-ESO-1 monoclonal antibody from ZyMed (San Diego Diego, South CA, USA). Western blotting kit was obtained from Pierce (Rockford, IL, USA), and BCA protein assay kit was from Puli Lai Gene Technology Co., Ltd (Beijing, China).

Instruments

Instruments used in this study included a Himac-CP70G low-temperature ultra-high speed centrifuge and a Hitachi TEM H-7500 transmission electron microscope (Hitachi Corporation, Tokyo, Japan). Electrophoresis devices used in this study included an electrophoresis tank and a transmembrane tank (Beijing 61 Instrument Factory, China), a GelDoc2000 gel imager (Bio-Rad Corporation, Chicago, IL, USA), a 100 ku MWCO Centriplus centrifugal ultrafiltration tube and a 100 ku MWCO Millipore Amicon high recovery-high-flow tangential flow ultrafiltration centrifuge tube (Millipore Corporation, Bedford, MA, USA).

Cell culture

Human hepatoma cell lines, HepG2 and Hep3B, were maintained at 37℃ in 10% DMEM containing 10% FCS (Gibco Corporation, Carlsbad, CA, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma Corporation, Santa Clara, CA, USA). HepG2 and Hep3B cells were divided into 3 control groups and 3 experimental groups, respectively, for regular culture. Cell viability was 95% as determined by trypan blue exclusion. Twenty-four hours after inoculation, cells in experimental groups were treated with 5-aza-CdR at a concentration of 1×10^{-6} mol/L and 150 mL of culture supernatant was collected 72 h later from each group, while cells in control groups were cultured without any drug and 150 mL of culture supernatant was collected from each group as controls.

Isolation and purification of exosomes

Exosomes were isolated as previously described[18]. In brief, 150 mL of a medium from confluent cultures (5-7 d) was harvested and centrifuged twice (2000 *g* and 10000 *g*) to remove cells and debris. Clarified supernatant was then ultrafiltrated using 100 ku MWCO Centriplus centrifugal ultrafiltration tubes to remove big-molecule compounds and reduce the volume of samples before ultracentrifugation on a 30% sucrose/D_2O cushion, which was collected and diluted in PBS. Ultrafiltration was then performed using a 100 ku MWCO Millipore Amicon high recovery-high-flow tangential flow ultrafiltration centrifuge tube. Finally, 5 mL of exosomes was obtained and stored at 4℃ for no more than 48 h before use.

Reverse transcription polymerase chain reaction

Total RNA was extracted from HepG2 and Hep3B cells using a Promega's total RNA extraction kit 72 h before and after 5-Aza-Cdr treatment. The sequences of p53 upstream and downstream primers used in this study are 5'-ACCCAGGTCCAGATGAAG-3' and 5'-CACTCGGATAAGATGCTGA-3', respectively. The length of amplified fragments was 422 bp. The sequences of β-actin upstream and downstream primers used in this

study are 5'-CGGGAAATCGTGCGTGACATT-3' and 5'-GGAGTTGAAGGTAGTTTCGTGG-3', respectively. The length of amplified fragments was 150 bp. RT reaction system contained 2 μg total RNA, 150.5 μg oligo (dT), 5 × PCR buffer, 1 mmol/L dNTP, 20U RNasin, 200 U M-MLV reverse transcriptase. PCR system contained 2 μL reverse transcriptase reaction products, 500 pmol/L of each upstream and downstream primer, 200 μmol/L of each dNTP, 1 U Taq DNA synthesis enzyme, and 10 × PCR buffer. The total reaction volume was 20 μL. Thirty cycles of PCR amplification were performed under the following conditions: predenaturation at 94℃ for 5 min, denaturation at 94℃ for 30 s, annealing at 55℃ for 30 s, extension at 72℃ for 30 s, and a final extension at 72℃ for 5 min. Finally, 6 uL of PCR products was electrophoresed on 1.5% agarose gel. The results were recorded using a gel imaging system for semi-quantitative analysis by grey ratio of target gene to β-actin. Reverse transcription polymerase chain reaction (RT-PCR) reagents were purchased from Promega Company (San Luis Obispo, CA, USA) and primers were synthesized in Beijing Bioko Biotech Co., Ltd (Beijing, China).

Marking and counting exosomes under colloidal gold immunoelectron microscope

An antigen-antibody complex was prepared. In brief, 10 μL of exosomes was obtained and mixed with an equal volume of rabbit anti-human HSP70 monoclonal antibody (1:50 dilution), mouse anti-human HLA- I monoclonal antibody (1:100 dilution) or mouse anti-human NY-ESO-1 monoclonal antibody (1:50 dilution), respectively. These samples were dropped onto a copper mesh surface and incubated for 1 h at room temperature. Twenty μL of SPA diluted at 1:15 was dropped onto the hydrophobic membrane to form liquid beads. The copper mesh was floated in SPA droplets with its membrane surface faced down at room temperature for 30 min. Then, 15 μL of uranyl acetate drops was put onto the copper mesh surface and stained at room temperature for 30 s. A SPA-coated copper mesh was taken as a control. Exosomes with black colloidal gold particles on the capsular membrane and cavity were marked as positive under transmission electron microscope.

Quantification of exosome immune molecules under electron microscope

Exosomes and their labeled colloidal gold particles were counted under each camera view of exosome samples. Each protein was represented by the number of colloidal gold particles in 100 exosomes. The data were expressed as mean ± SD for further statistical analysis.

Exosome counting under electron microscope

The area under each photographic view was 1000 nm × 700 nm. The number of exosomes under each field of vision was counted. The average number of exosomes under each camera view was calculated in 10 randomly selected horizons. The number of exosomes in each mL supernatant of the cells was figured out by copper mesh diameter (2.5 mm) and area (1.25 mm × 1.25 mm × 3.14). The cells in each of experimental and control groups were counted three times and averaged for statistical analysis.

Western blotting

Forty microgramme of exosomes was taken and 10% SDS-PAGE electrophoresis was performed at a constant electric power. After electrophoresis, the gels were transferred to NC membrane, incubated with HSP70, HLA- I and NY-ESO-1 antibodies diluted at 1:500 overnight at 4℃. Then, horseradish peroxidase-tagged antibody was added and incubated at room temperature for 2 h. Photography was performed with a Kodak X-OMAT film (Eastman Kodak, Rochester, NY). The light absorption value *A* of target bands and β-actin was determined using the imaging analysis system. The results were indicated by the absorption ratio of target bands and β-actin, and averaged from three independent experiments.

Determination of protein concentration in exosomes

Exosomes in two cell lines of experimental and control groups were taken to determine the protein concentration with a bicinchoninic acid (BCA) protein assay kit following its manufacturer's instructions.

Statistical analysis

The data were expressed as mean ± SD. Statistical analysis was performed using the SPSS13.0 statistical software. $P < 0.05$ was considered statistically significant.

RESULTS

Exosomes and their related immune molecules identified by immunoelectron microscopy

Immunoelectron microscopy showed that exosomes, secreted from HepG2 and Hep3B cells, had the membranous structure of microcapsules, 30-80 nm in diameter. They were round or oval and their cavities were full of components with a low electron density. After colloidal gold immunoelectron marking, dotted, granular colloidal gold markers could be observed in the capsular membranes and cavities. Each kind of immune molecules had no more than two gold-labeled particles in the control groups. However, after treatment with 5-Aza-CdR, 5, 4, and 3 gold-labeled HSP70, HLA- I and NY-ESO-1 molecules were observed in exosomes, respectively. The number of gold-labeled particles in each immune molecule was significantly greater in experiment groups than in control groups ($P < 0.05$, Figure 1, Table 1).

Tumor antigen and immune molecules in exosomes detected by Western blotting

In the supernatant of exosomes from two cell lines, a clear specific protein band could be detected by Western blotting at the molecular weights of 73, 40 and 18 kDa. After treatment with 5-Aza-CdR, the HLA- I and NY-ESO-1

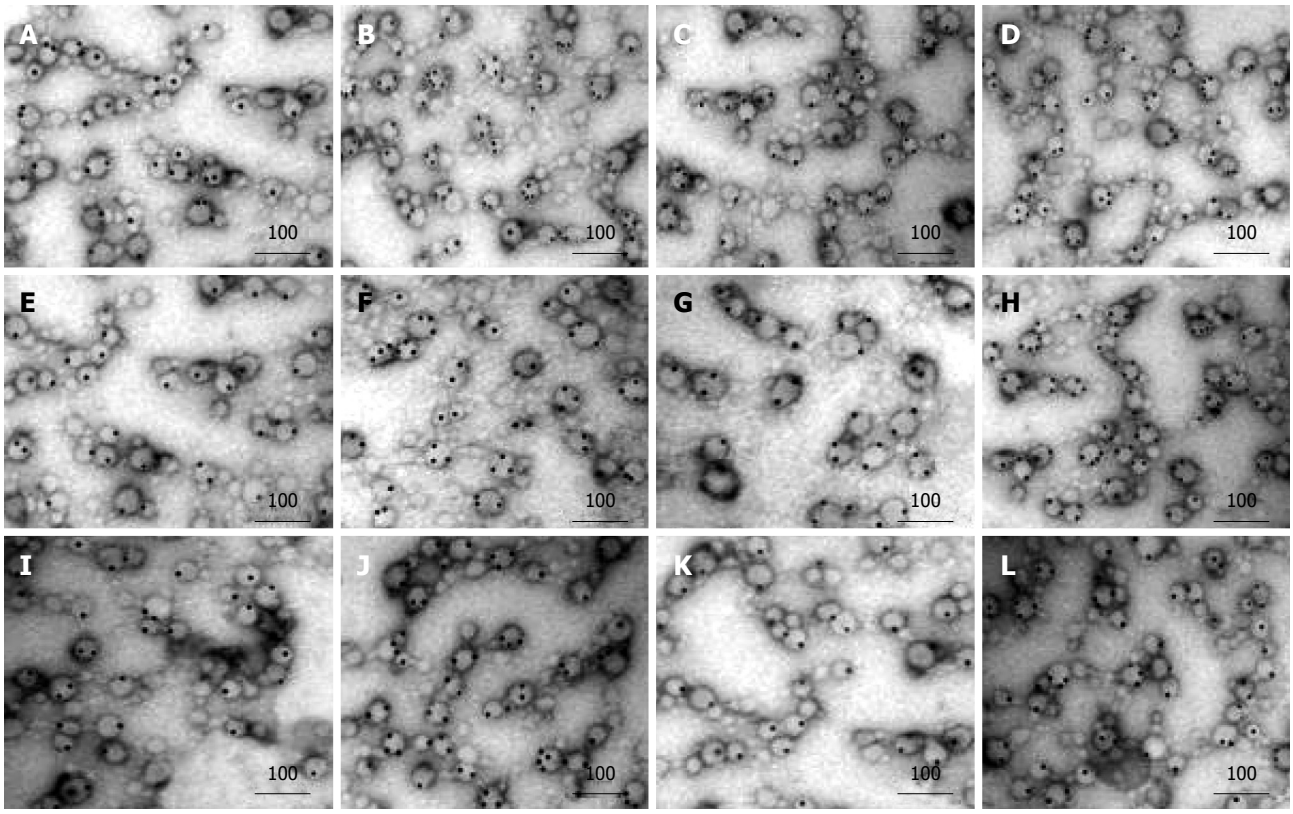


Figure 1 Immunoelectron microscopy showing colloidal gold HSP70 labeled HepG2 exosomes in control group (A) and in experiment group (B), colloidal gold HLA-Ⅰ labeled HepG2 exosomes in control group (C) and experiment group (D), colloidal gold NY-ESO-1 labeled HepG2 exosomes in control group (E) and experiment group (F), colloidal gold HSP70 labeled Hep3B exosomes in control group (G) and experiment group (H), colloidal gold HLA-Ⅰ labeled Hep3B exosomes in control group (I) and experiment group (J), colloidal gold NY-ESO-1 labeled Hep3B exosomes in control group (K) and experiment group (L) (× 140 000).

Table 1 Immune-related molecules of exosomes in control and experimental groups (mean ± SD)

	n	HepG2		*P*	Hep3B		*P*
		Control group	Experiment group		Control group	Experiment group	
HSP70	3	74.2 ± 3.1	99.8 ± 5.2	< 0.01	70.2 ± 5.1	99.9 ± 3.8	< 0.01
HLA-Ⅰ	3	53.3 ± 2.5	79.6 ± 6.1	< 0.01	40.6 ± 2.7	86.7 ± 3.6	< 0.01
NY-ESO-1	3	38.2 ± 4.2	58.2 ± 4.3	< 0.01	32.1 ± 4.6	70.3 ± 2.9	< 0.01

expression level was significantly higher in experimental groups than in control groups ($P < 0.05$), while the HSP70 expression did not increase significantly (Figure 2).

Exosomes and protein in exosomes under electron microscope

The number of exosomes secreted from both cell lines and the total protein in exosomes were significantly higher after treatment with 5-Aza-CdR than before treatment with 5-Aza-CdR ($P < 0.05$, Table 2).

Change of wild-type P53 gene expression in HepG2 and Hep3B cells after 5-Aza-CdR treatment

The *p53* gene was moderately expressed in HepG2 cells but not in Hep3B cells before 5-Aza-CdR treatment. However, the *p53* gene mRNA was significantly expressed in two cell lines after 5-Aza-CdR treatment (Figure 3).

DISCUSSION

5-Aza-CdR can recover some genes with CpG islands in their promoter regions, or significantly increase the expression of these genes by directly inhibiting the DNA methyltransferase activity[19]. It has been shown that DNA methylation can regulate cancer/testis antigen (CTA), MHC Ⅰ, MHC Ⅱ, and a variety of immune adhesion molecules[20,21]. 5-Aza-CdR can significantly increase their expression in tumor cells and improve anti-tumor immune response capacity[17].

Exosomes, originating as vesicles in some late endosomes, would release when these mature endosomes or multivesicular bodies (MVBs) are fused with cell membranes. Exosomes carry a variety of specific proteins, such as antigen-presenting-associated protein, T cell activation-associated protein, and some tumor antigens and antigen

Table 2 Exosomes and their total protein level in hepatoma cells (mean ± SD)

Subject	*n*	HepG2 con	HepG2 exp	*P*	Hep3B con	Hep3B exp	*P*
Exo No	3	$3.56 \times 10^8 \pm 2.6 \times 10^2$	$4.53 \times 10^9 \pm 3.7 \times 10^2$	0.010	$3.12 \times 10^8 \pm 6.2 \times 10^2$	$4.13 \times 10^9 \pm 3.7 \times 10^2$	0.021
Exo Pro	3	0.92 ± 0.13 mg	1.31 ± 0.16 mg	0.012	0.89 ± 0.10 mg	1.52 ± 0.16 mg	0.011

Exo: Exosome; con: Control group; exp: Experiment group.

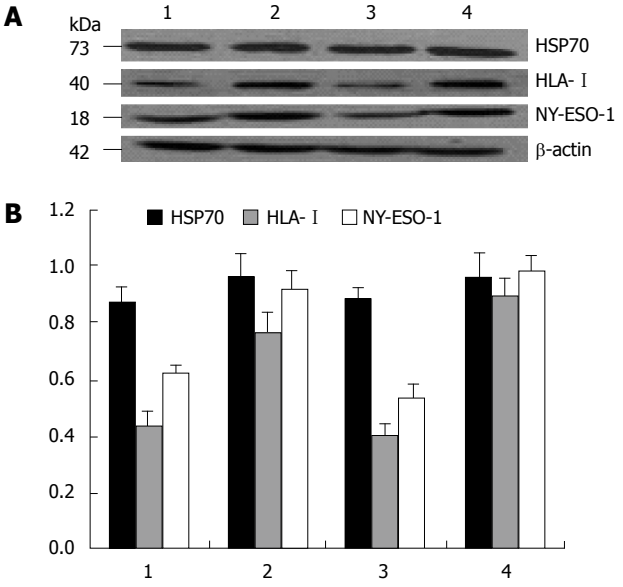


Figure 2 Immune molecules Western blotting results (A), semi-quantities results of Western blotting (B). 1: HepG2 control; 2: HepG2 experiment; 3: Hep3B control; 4: Hep3B experiment.

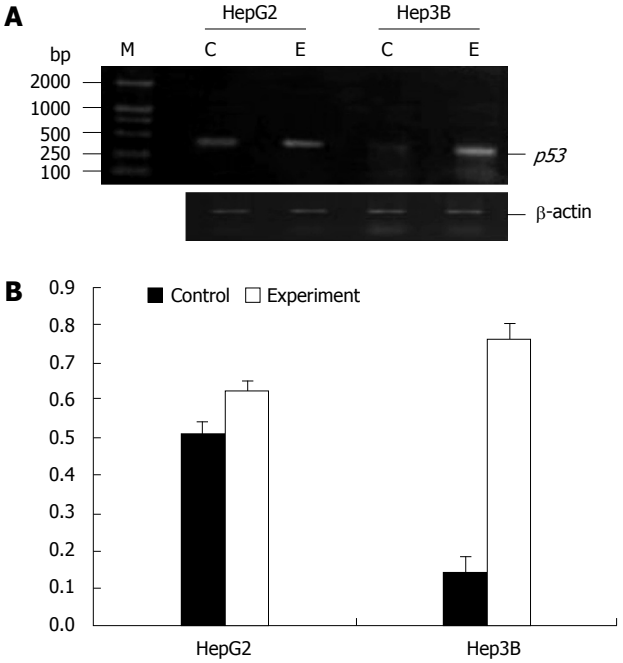


Figure 3 RT-PCR results of *p53* gene expression (A), semi-quantities results of RT-PCR (B). M: Marker; C: Control group; E: Experiment group.

chaperones[7]. Exosomes themselves offer an antigen delivery system and can transfer tumor antigens to antigen-presenting cells, causing anti-tumor responses[22].

For these reasons, use of exosomes in immunotherapy and research requires high-quality exosomes. However, the mechanism underlying exosome secretion has not been well elucidated. Yu *et al*[11] found that activation of wild-type p53 increases exosome production by upregulating a transmembrane protein, TSAP6, which selectively transports proteins to exosomes and adjusts exosome formation. 5-Aza-CdR can increase *p53* gene expression by damaging or methylating DNA[12,14]. In addition, it has been reported that 5-Aza-CdR increases the expression of CTA, HLA- I , and HLA- Ⅱ through demethylation, thus improving anti-tumor specific immune response and decreasing the size of transplanted tumors in mice[15,16]. Therefore, this study investigated whether 5-Aza-CdR can increase the number of exosomes and the immune molecule level in exosomes secreted by hepatoma cells through *p53* gene activation and DNA demethylation pathways.

Immunoelectron microscopy and Western blotting in this study showed that exosomes derived from HepG2 and Hep3B cells were rich in HSP70 protein both before and after 5-Aza-CdR treatment. However, after 5-Aza-CdR treatment, electron microscopy showed that the tagged HSP70 proteins in exosomes were increased, while Western blotting showed that they did not, possibly due to the fact that *HSP70* gene expression is not regulated by DNA methylation. Moreover, HLA- I and NY-ESO-1 molecules were clearly regulated by DNA methylation, the expression of these molecules especially that of HLA- I in Hep3B cells, was relatively low in control groups. After 5-Aza-CdR treatment, these molecules were significantly increased in both cell lines. The number of exosomes secreted by treated and untreated hepatoma cells was calculated and changes of protein in exosome stock solutions treated and untreated with BCA were observed under electron microscope, showing that after 5-Aza-CdR treatment, the number of exosomes secreted by hepatoma cells and the protein in exosomes are significantly increased ($P < 0.05$). Furthermore, immune electron microscopy and Western blotting revealed that the number of exosomes was increased by almost an order of magnitude. Although there was a difference in *p53* mRNA expression between the two hepatoma cell lines before treatment with 5-Aza-CdR, the *p53* expression was significantly increased in both cell lines, especially in Hep3B cell line after 5-Aza-CdR treatment,. The number of exosomes and the expression of HSP70 protein were slightly higher in Hep3B cells than in HepG2 cells. However, the expression of HLA- I and NY-ESO-1 molecules was significantly higher in Hep3B cells than in HepG2 cells, indicating that exosomes secreted by hepatoma cells are regulated by the *p53* gene, while

HLA-Ⅰ and NY-ESO-1 proteins in exosomes are mainly regulated by DNA methylation. As a "molecular chaperone", HSP70 can actively deliver antigens through receptors on antigen-presenting cell membranes, thus increasing the efficiency of antigen presentation by 104-folds of pure pinocytosis or phagocytosis. Tumor-specific antigens are released from HSP within cells and presented to cytotoxic T cells, which are activated to execute immune responses to different tumors[23]. NY-ESO-1, belonging to the CTA family, is expressed in a variety of tumor tissues. Anti-NY-ESO-1 specific antibodies and sensitized lymphocytes can be found in serum of hepatoma patients[24,25]. Thus, NY-ESO-1 is currently thought to be a tumor-specific antigen with the strongest antigen immunogenicity[26]. However, NY-ESO-1 re-expression in tumor cells is not homogeneous and its low expression level is insufficient to stimulate cell-mediated immunity. To enhance tumor-specific immunity, sufficient tumor-specific antigens or immune-associated molecules are needed.

In conclusion, hepatoma cells secrete more exosomes after 5-Aza-CdR treatment. Moreover, the expression of tumor-specific antigen, NY-ESO-1, and tumor-specific immune stimulating molecule, HLA-Ⅰ, increase significantly. While 5-Aza-CdR up-regulates *p53* gene expression, it may play a more important role in DNA demethylation. Exosomes secreted from hepatoma cells after 5-Aza-CdR treatment are more able to stimulate anti-tumor-specific immune response, which may be useful for the preparation of a new cancer therapeutic vaccine for hepatoma.

COMMENTS

Background

Human hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. However, no effective curative therapy for it is currently available. It is therefore necessary to develop a novel strategy against HCC. Tumor-derived exosomes, containing tumor-associated antigens and chaperones, are membrane nanoparticles, which make them stable, easy to maintain and susceptible to uptake by immune cells. However, cancer/testis antigens (CTAs) with a strong immunity and immune-associated molecules with adjuvant anti-cancer effects are heterogeneous and insufficient in cancer cells to emit specific immune reactions. Therefore, getting enough quantity and quality of exosomes for a sufficient immune effect remains a great challenge for tumor immunotherapy. 5-Aza-CdR, an inhibitor of DNA methyltransferase, can restore or increase CTAs and HLA-Ⅰ/Ⅱ expression in cancer cells by demethylation and induce *p53* gene expression by damaging DNA, while p53 can increase exosome production. The authors hope to get more exosomes with a large number of CTAs and immune-associated molecules by modifying hepatoma cells with 5-Aza-CdR.

Research frontiers

Exosomes secreted by tumor or dendritic cells are a recent hot spot in tumor immunology research. However, insufficient tumor-associated antigens and tumor-derived immune stimulated molecules are still a problem in such a research. Various methods, such as *IL-2* gene transfection and curcumin-modified exosomes, have been used to improve exosome immune stimulation. In this study, hepatoma was treated with 5-Aza-CdR, and more powerful exosomes were produced.

Innovations and breakthroughs

5-Aza-CdR, a DNA methyltransferase inhibitor and a demethylation promoter of CpG in many genes including the *p53* gene, can significantly restore or increase their expression. It has been shown that 5-Aza-CdR can significantly increase the expression of molecules necessary for anti-tumor cellular immunity such as HLA-Ⅰ and HLA-Ⅱ, and the therapeutic effect of anti-tumor immunity *in vitro* and in animal experiments. However, no report is available on the effect of 5-Aza-CdR on the number of exosomes in cancer cells. This study showed that 5-Aza-CdR could increase exosomes in hepatoma cells and immune-related molecules of exosomes. Although its mechanism remains unclear, it may be related to demethylation and the p53-induction effect of 5-Aza-CdR. These findings suggest that 5-Aza-CdR can modify exosomes, thus offering an interesting possibility for developing a cancer vaccine.

Applications

This study has confirmed that 5-Aza-CdR-modified exosomes from hepatoma cells contain more CTAs, chaperones and immune-stimulating molecules, which may be used in preparation of a new cancer therapeutic vaccine *in vitro* for hepatoma.

Terminology

Exosomes, secreted by many kinds of cells including blood and tumor cells, have a membrane structure, 30-100 nm in size, consisting of proteins and RNA, and function as a vehicle for messages from cell to cell.

Peer review

The authors studied the effect of 5-Aza-CdR on the number of exosomes and immune-associated proteins in them produced by hepatoma cell lines, HepG2 and Hep3B, showing that exosomes function as a vehicle for messages from cell to cell, which may be used in preparation of a new cancer therapeutic vaccine for hepatoma.

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ORIGINAL ARTICLE

Chronic niacin overload may be involved in the increased prevalence of obesity in US children

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Abstract

AIM: To investigate nicotinamide's action on glucose metabolism, and the association between niacin consumption and obesity prevalence.

METHODS: Dynamic nicotinamide's effect on plasma hydrogen peroxide and glucose metabolism was investigated using oral glucose tolerance tests with or without nicotinamide in the same five healthy subjects. Lag-regression analysis was used to examine the association between the niacin consumption and the obesity prevalence among US children using the data from the Economic Research Service of the US Department of Agriculture and from US Centers for Disease Control and Prevention, respectively.

RESULTS: Compared with the control oral glucose tolerance test, the 1-h plasma hydrogen peroxide (1.4 ± 0.1 μmol/L *vs* 1.6 ± 0.1 μmol/L, $P = 0.016$) and insulin levels (247.1 ± 129.0 pmol/L *vs* 452.6 ± 181.8 pmol/L, $P = 0.028$) were significantly higher, and the 3-h blood glucose was significantly lower (5.8 ± 1.2 mmol/L *vs* 4.5 ± 1.1 mmol/L, $P = 0.002$) after co-administration of glucose and 300 mg nicotinamide. The obesity prevalence among American children increased with the increasing per capita niacin consumption, the increasing grain contribution to niacin due to niacin-fortification, and the increasing niacin-fortified ready-to-eat cereal consumption, with a 10-year lag. The regression analyses showed that the obesity prevalence in the US children of all age groups was determined by niacin consumption ($R^2 = 0.814$, 0.961 and 0.94 for 2-5 years, 6-11 years and 12-19 years age groups, respectively).

CONCLUSION: The appetite-stimulating effect of nicotinamide appears to involve oxidative stress. Excess niacin consumption may be a major factor in the increased obesity prevalence in US children.

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Key words: Obesity; Diabetes; Niacin; Nicotinamide

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INTRODUCTION

The high prevalence of obesity, a major risk factor for type 2 diabetes, has been recognized as a serious global health problem[1]. It is likely that both dietary factors and physical inactivity may contribute to the development of obesity[2,3]. However, the reason why the prevalence of obesity suddenly increased dramatically starting around 1980 in the US is not well understood[4]. It is argued that eating and dietary quality factors may act as a primary driver for the obesity epidemic[2,4]. Therefore, exploring dietary risk factors should be of importance for the prevention and treatment of obesity and type 2 diabetes.

During the past few decades, one of the significant, but relatively overlooked worldwide changes in dietary composition has been the marked increase in the content of niacin (nicotinamide and nicotinic acid). For example, as shown in Figure 1A, the daily US per capita niacin consumption has maintained an increasing trend since the early 1940s, and has reached 33 mg in the early 2000s[5], which is more than two times the recommended dietary allowance (RDA) by the US Food and Nutrition Board (RDA: 14 and 16 mg/d for adult women and men, respectively)[6]. Animal flesh (meat, poultry and fish) and grains are the largest contributors of dietary niacin. Most of the increased niacin consumption comes from grain products due to the implementation of mandatory niacin-fortification (i.e. the addition of niacin to food) started from the early 1940s[7]. Most significantly, a sharp increase in the grain contribution in 1974 due to an update of niacin-fortification standards has made grains the largest contributor (Figure 1B)[8,9]. However, the effect of long-term exposure to excess niacin on human health is poorly understood.

Niacin-fortification has been practiced first in many developed countries to prevent pellagra, a disease due to niacin deficiency[10], and then introduced to developing countries[11]. Soon after the introduction of niacin-fortification, the prevalence of obesity and diabetes has begun to increase rapidly first in developed countries and then in developing countries[12,13], basically in a similar way as the niacin-fortification spread. Thus, there is the possibility that niacin-fortification-induced increase in dietary niacin may be involved in the increased global prevalence of obesity and diabetes. However, there are very limited data regarding the possible adverse long-term consequences of niacin-fortification.

Grains (flour and cereals), the main source of carbohydrate, are the most widely used vehicles for niacin-fortification[11]. As a result, the niacin content in the fortified grain products has been significantly increased. Traditionally, low-fat, high-carbohydrate diets were used to treat type 2 diabetes[14,15]. However, since the implementation of niacin-fortification in the US from the early 1940s and the significant increase in carbohydrate consumption during the past three decades, more and more studies from the US have found that high-carbohydrate diets increase, instead of decrease, the risk for obesity and type 2 diabetes[12,16,17]. Then, low-carbohydrate diets have been used to treat obesity and diabetes since the late 1990s[18-20]. Whether such a profound change in the effect of carbohydrate diets is related to niacin fortification of grains is not known.

Obesity is characterized by increased appetite and insulin resistance[21,22], whereas niacin is a potent stimulator of appetite and niacin deficiency may lead to appetite loss[10]. Moreover, large doses of niacin have long been known to impair glucose tolerance[23,24], induce insulin resistance and enhance insulin release[25,26]. Evidence suggests that niacin-induced increase in insulin release may be a compensation of pancreatic islet β cells in response to the insulin resistance[25,26]. However, whether excess niacin intake is involved in the increased appetite and the insulin resistance of obesity is unclear. Our recent study found that oxidative stress may mediate excess nicotinamide-induced insulin resistance, and that type 2 diabetic subjects have a slow detoxification of nicotinamide. These observations suggested that type 2 diabetes may be the outcome of the association of high niacin intake and the relative low detoxification of niacin of the body[27]. Based on these lines of evidence, we postulated that excess niacin intake may also play a role in obesity. To address this issue, this study explored the mechanism underlying niacin's action on glucose metabolism, and the association between the US per capita niacin consumption and the obesity prevalence in the US.

MATERIALS AND METHODS

Nicotinamide load test

The present study was approved by the relevant ethics committee, and all the participants gave informed consent. Five healthy young male volunteers aged 20-24 years participated in the two oral glucose tolerance tests (glucose dose 75 g) with (NM-OGTT) or without (C-OGTT) oral co-administration of 300 mg nicotinamide (Lisheng Pharma, Tianjin, China), respectively, with an interval of 4 d. Each test was conducted after an overnight fast, venous blood (1.8 mL) was collected into sodium citrate tubes before, and 1, 2, 3, and 4 h after the administration. The blood samples were separated by centrifugation (1500 × *g*, 10 min). Aliquots of each plasma sample were placed directly in liquid nitrogen and then transferred to -80℃ for later analysis.

Assays of blood glucose, plasma insulin and hydrogen peroxide

Blood glucose was measured using a glucometer (One-Touch Ultra, LifeScan Inc.). Plasma insulin was measured by radioimmunoassay using commercial kits (Beijing North Institute of Biological Technology, China). Plasma hydrogen peroxide (H_2O_2) concentration was measured using an H_2O_2 Assay Kit (Beyotime Biotechnology, Jiangsu, China).

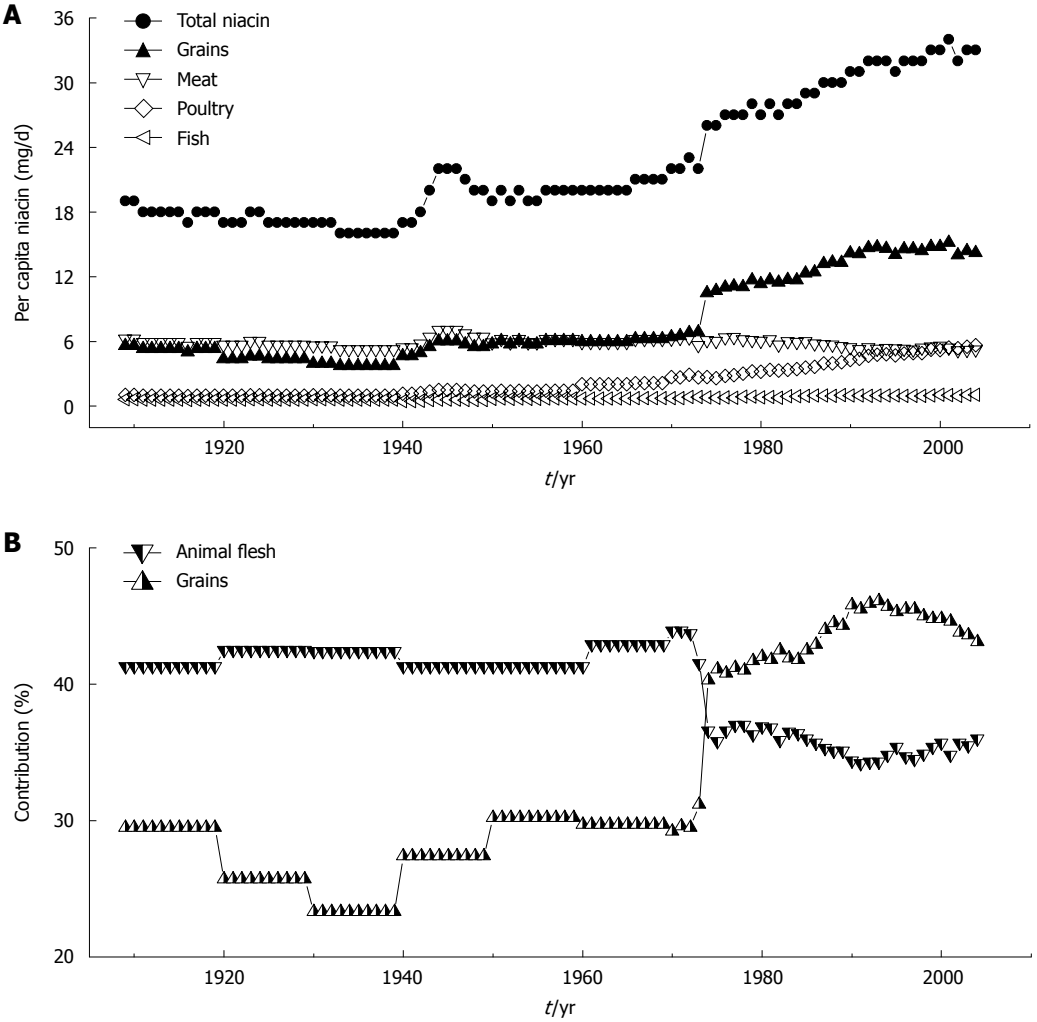


Figure 1 Trends in per capita niacin consumption and contributions of grains and animal flesh to niacin in the US. A: The trends in total niacin consumed per capita per day in 1909-2004 and in the amounts from grains and animal flesh (meat, poultry and fish); B: Changes in the percentage of grain and animal flesh contribution to dietary niacin. The data on niacin consumption are derived from Ref. 5. The data on the grain and animal flesh contribution to niacin are derived from Ref. 8 (1909-2000) and Ref. 9 (2001-2004). The per capita niacin consumption (A) and the grain contribution to niacin (B) have been suddenly increased since 1974 due to an update in niacin-fortification standards.

Determination of nicotinamide and N^1-methylnicotinamide

Nicotinamide and N^1-methylnicotinamide were analyzed, as previously described[27], using a high-performance liquid chromatography (HPLC) system that consisted of an LC-9A pump (Shimadzu, Kyoto, Japan), a Rheodyne 7725i sample injector with a 20-μL sample loop (Rheodyne LLC, Rohnert Park, CA, USA), a Hypersil ODS C18 column (Thermo, Bellefonte, PA, USA) and a Waters 470 fluorescence detector (Milford, MA, USA).

Data sources

The data used for assessing the relationship between niacin consumption and obesity prevalence were derived from the databases of the US Centers for Disease Control and Prevention (CDC) and of the Economic Research Service of the US Department of Agriculture. The data on the prevalence of obesity (body mass index ≥ 95th percentile for age and sex) in US children and adolescents were from National Health Examination Survey (NHES) 2 (1963-1965, only available for the age group of 6-11 years), NHES 3 (1966-1970, only available for the age group of 12-19 years), the National Health and Nutrition Examination Surveys (NHANES) I (1971-1974), Ⅱ (1976-1980), Ⅲ (1988-1994), and the continuous NHANES data collection (1999-2000, 2001-2002, 2003-2004)[28-30]. NHES and NHANES are conducted by the CDC, and include a series of cross-sectional nationally representative health examination surveys. Each cross-sectional survey provides a national estimate for the US population at the time of the survey. Detailed descriptions of the survey methods are available elsewhere[29,30], and on-line (http://www.cdc.gov/nchs/nhanes.htm). The nutrient data on the US per capita niacin consumption in 1909-2004[5], grain consumption in 1909-2007[31], grain contribution to niacin in 1909-2000[8] and in 2001-2004[9] and ready-to-eat cereal (RTE) consumption[32] were derived from the databases of the Economic Research Service (ERS) of the US Department of Agriculture. ERS annually calculates the amounts of several hundred foods

available for human consumption in the US and provides estimates of per capita availability. The estimates of nutrients in the food supply reflect Federal enrichment and fortification standards and technological advances in the food industry[8].

Statistical analysis

The data are presented as mean ± SD. Statistical differences in the data were evaluated by paired Student's *t* test. Lag-regression analysis was used to test the relationships between the consumption of niacin or grain and the obesity prevalence in the US children and adolescents using SPSS software (SPSS Inc., Chicago, USA). Statistical significance was set at $P < 0.05$.

RESULTS

Dynamic effect of nicotinamide on blood glucose metabolism

The methylation of nicotinamide to N^1-methylnicotinamide is a major mechanism to eliminate excess nicotinamide[27]. As shown in Figure 2, in C-OGTT, the plasma concentrations of nicotinamide and N^1-methylnicotinamide were fairly constant (Figure 2A and B), and the 2-h plasma level of H_2O_2, a major reactive oxygen species (ROS), was slightly increased, but without statistical significance compared with the value before C-OGTT (Figure 2C). In contrast, NM-OGTT led to a significant increase in plasma nicotinamide and N^1-methylnicotinamide levels, with a peak at approximate 2 h (Figure 2A and B). In parallel with the rising phase of plasma nicotinamide and N^1-methylnicotinamide, there was a significant increase in 1-h and 2-h plasma H_2O_2 levels in NM-OGTT (Figure 2C). Importantly, although the 1-h and 2-h blood glucose values were similar in the two tests (Figure 2E), however, the 1-h plasma insulin level was much higher in NM-OGTT than in C-OGTT (Figure 2D). As the plasma H_2O_2 returned to the basal level at 3 h in NM-OGTT (Figure 2C), there was a sharp decrease in the blood glucose concentration (Figure 2E). Two of the five subjects in NM-OGTT had reactive hypoglycemia symptoms (i.e. sweating, dizziness, faintness, palpitation and intense hunger) with the blood glucose levels below 3.6 mmol/L. In contrast, no subjects had reactive hypoglycemic symptoms during C-OGTT. These results indicated that nicotinamide overload might induce a biphasic effect, i.e. insulin resistance followed by hypoglycemia.

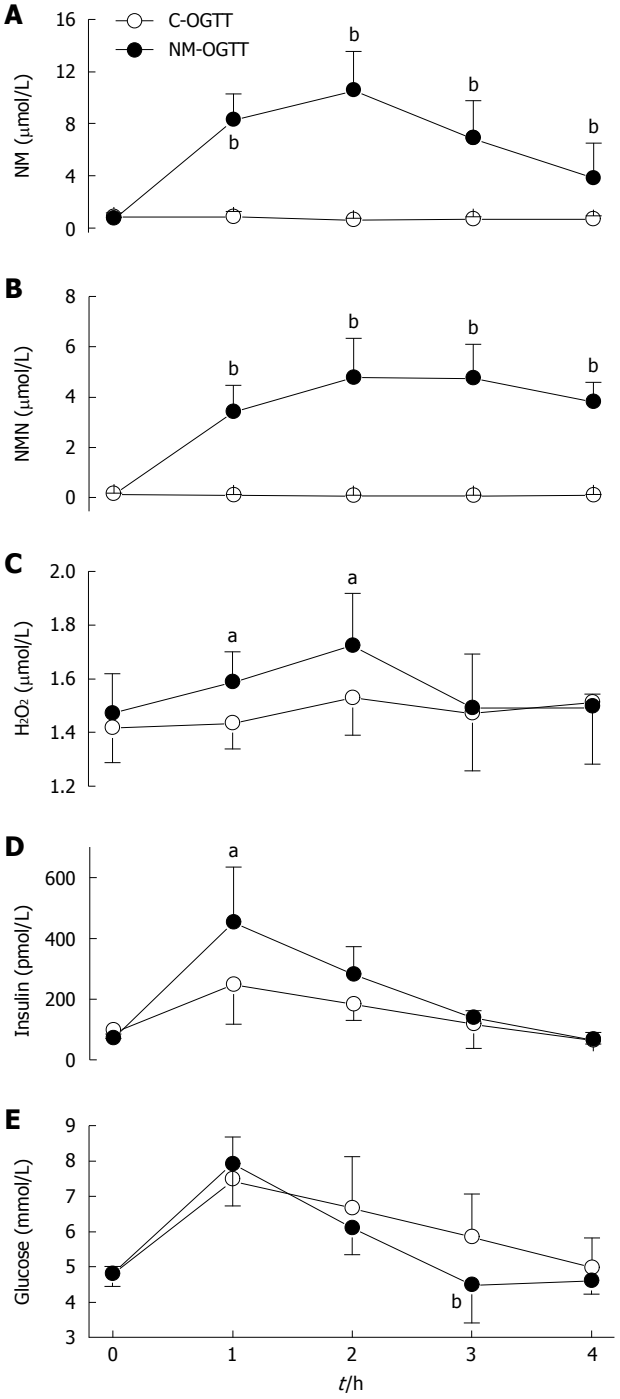


Figure 2 Dynamic effect of nicotinamide on plasma H_2O_2 level and glucose metabolism. A-E: Dynamic changes in the concentrations of plasma nicotinamide (NM), N^1-methylnicotinamide (NMN), H_2O_2, insulin and blood glucose in C-OGTT and NM-OGTT (co-administration of 300 mg nicotinamide and 75 g glucose). For each point, $n = 5$. Values represent mean ± SD, $^aP < 0.05$, $^bP < 0.01$ *vs* C-OGTT.

Association between niacin consumption and obesity prevalence in US children

We next investigated the relationship between the daily per capita niacin consumption and the obesity prevalence in US children and adolescents. There were two historical events in niacin-fortification in the US. The first is the initial introduction of niacin-fortification in the early 1940s and the second is the update of fortification standards in 1974. These two events resulted in significant increases in the per capita niacin consumption respectively in the 1940s and in the mid-1970s. Evidently, the prevalence of obesity in US children of all age groups increased in parallel with the increase in the per capita niacin consumption with a 10-year lag (Figure 3A, C and E). Lag-regression analysis revealed that the obesity prevalence in the children of all age groups was determined by niacin consumption (Figure 3B, D and F). This relationship was observed in both sexes (Figure 3G and H) (R^2 = 0.958 and 0.96 for boys and girls aged 6-11 years, P = 2.6e-17 and 1.6e-17,

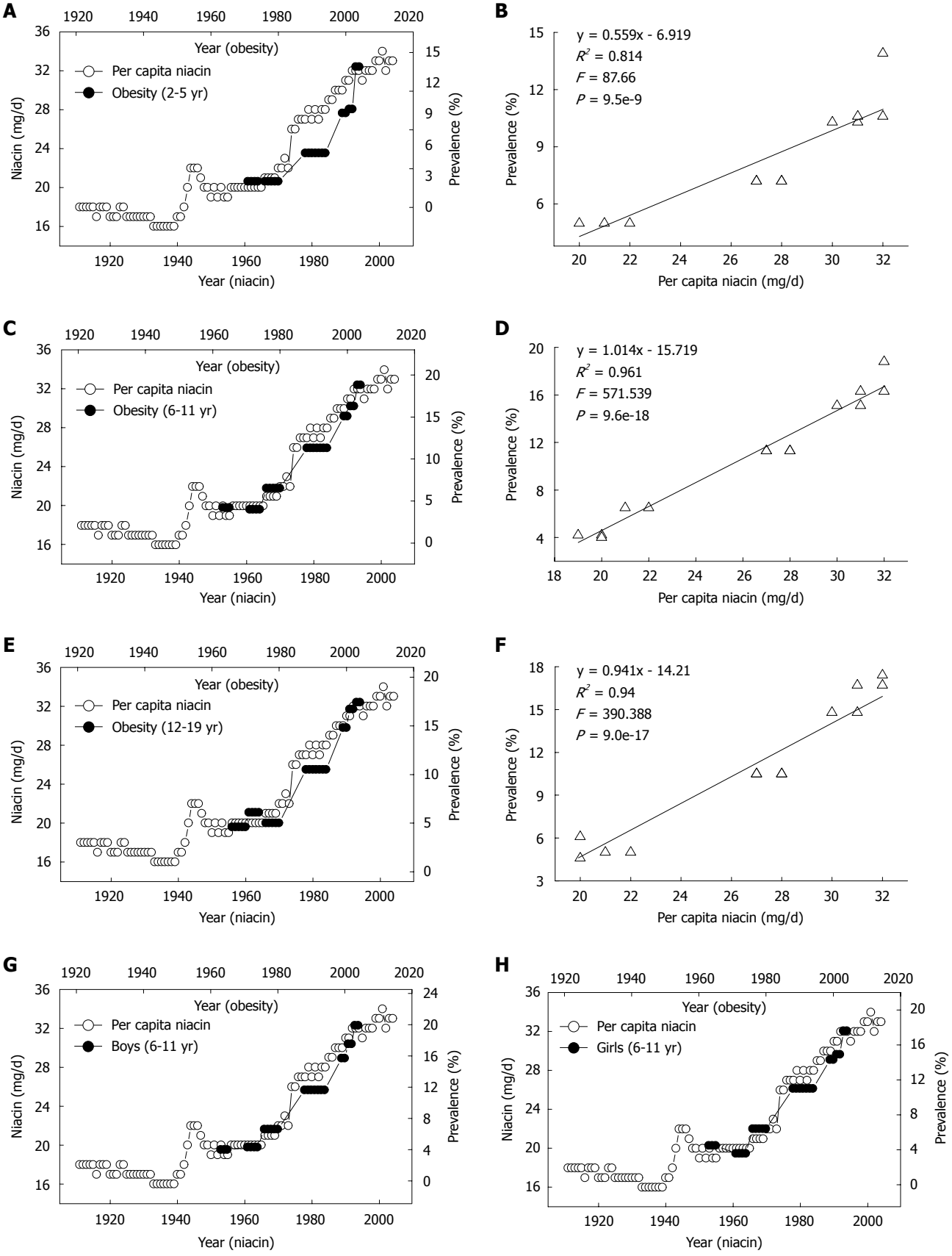


Figure 3 Correlations between US per capita niacin consumption and obesity prevalence in US children. A, C and E: The trends in the daily per capita niacin consumption in 1909-2004 (Ref. 5) and in the obesity prevalence in the children aged 2-5, 6-11 and 12-19 years (Ref. 28); B, D and F: The 10-year lag-regression plots of the obesity prevalence in different age groups against daily per capita niacin consumption using the data in A, C, and E; G and H: The obesity prevalence in the age group of 6-11 years of either sex in 1963-2004 (Ref. 29 and 30) increased in parallel with the per capita niacin consumption in 1953-1994.

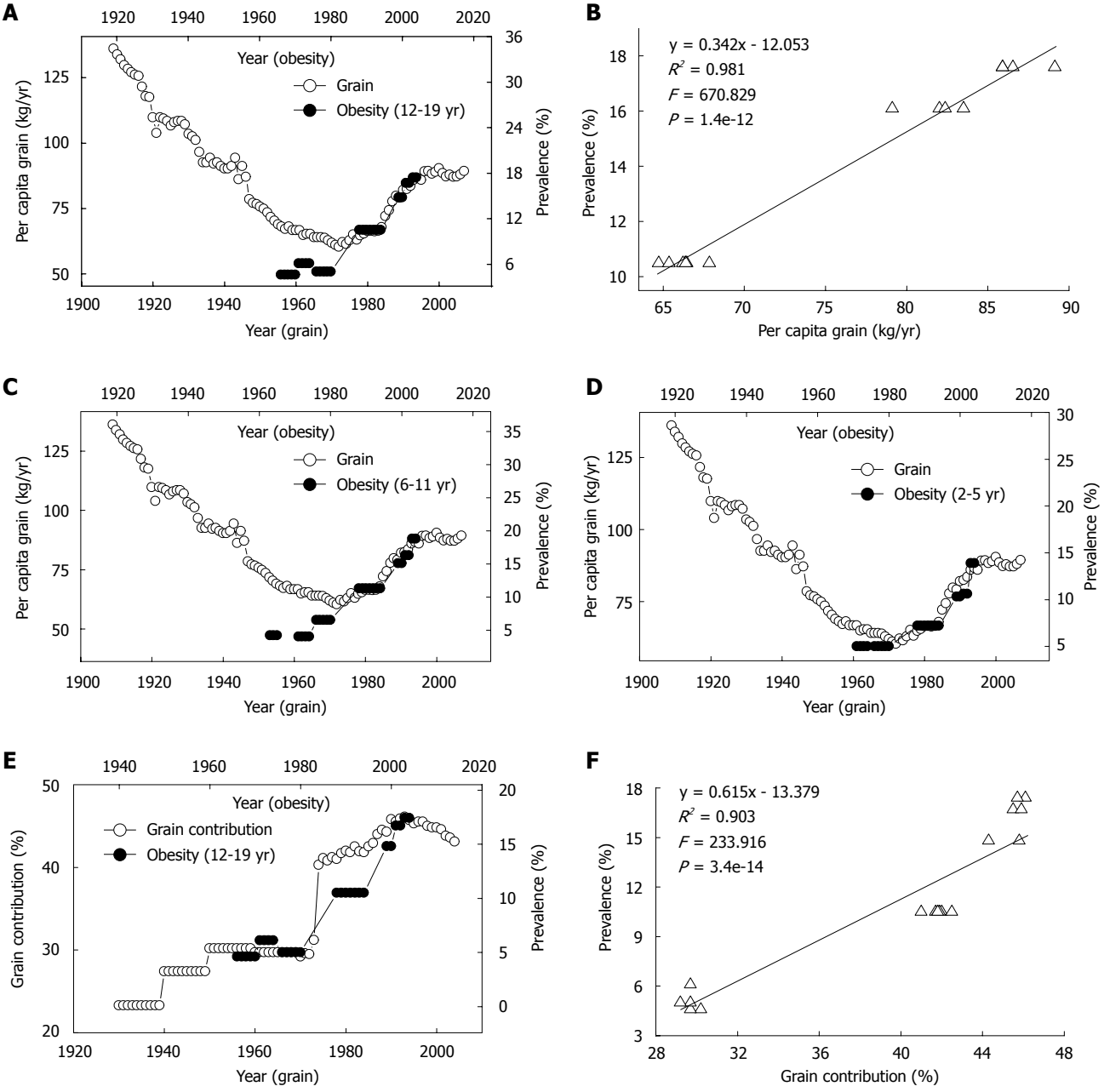


Figure 4 Correlations between consumption and contribution of grain and prevalence of obesity in US children. A: Trends in US per capita grain consumption in 1909-2007 (Ref. 31) and in the obesity prevalence in the children aged 12-19 years in 1966-2004 (Ref. 28); B: The linear regression plot of the obesity prevalence in 1988-2004 against grain consumption in 1978-1994 using the data in A; C and D: Relationships between the grain consumption (Ref. 31) and the prevalence of obesity in the children aged 6-11 and 2-5 years (Ref. 28). R^2 = 0.902 (P = 7.1e-7) and 0.955 (P = 9.7e-9), for 2-5 years age group and 6-11 years age group; E: Trends in the grain contribution to niacin (Ref. 8 and Ref. 9) and in the obesity prevalence in the children aged 12-19 years (Ref. 28); F: The linear regression plot of the prevalence of obesity in 1966-2004 against grain contribution in 1956-1994 using the data in E.

respectively; R^2 = 0.949 and 0.92 for boys and girls aged 12-19 years, P = 1.2e-17 and 3.4e-15, respectively).

Association between grain consumption and obesity prevalence in US children

Grains have been used as a major vehicle for niacin-fortification. The increase in the daily per capita niacin consumption from grains reflects the fortification level and the trend toward the consumption of fortified grain products. Although the US per capita grain consumption steadily decreased from the late 1930s through the early 1970s (Figure 4A), the grain contribution to niacin actually significantly increased due to niacin-fortification (Figure 4E). In the early 20th century, high per capita consumption of non-fortified grains was associated with a low prevalence of obesity in US children and adolescents. However, the re-increase in the consumption of the grain products fortified with more niacin since 1974 was followed by a steep increase in the obesity prevalence in the US children of all age groups in the 1980s and 1990s. The lag was ten years (Figure 4A, C and D). Moreover, the obesity prevalence in US children also increased in parallel with the increase in grain contribution to niacin with a 10-year lag (Figure 4E). The regression analyses showed

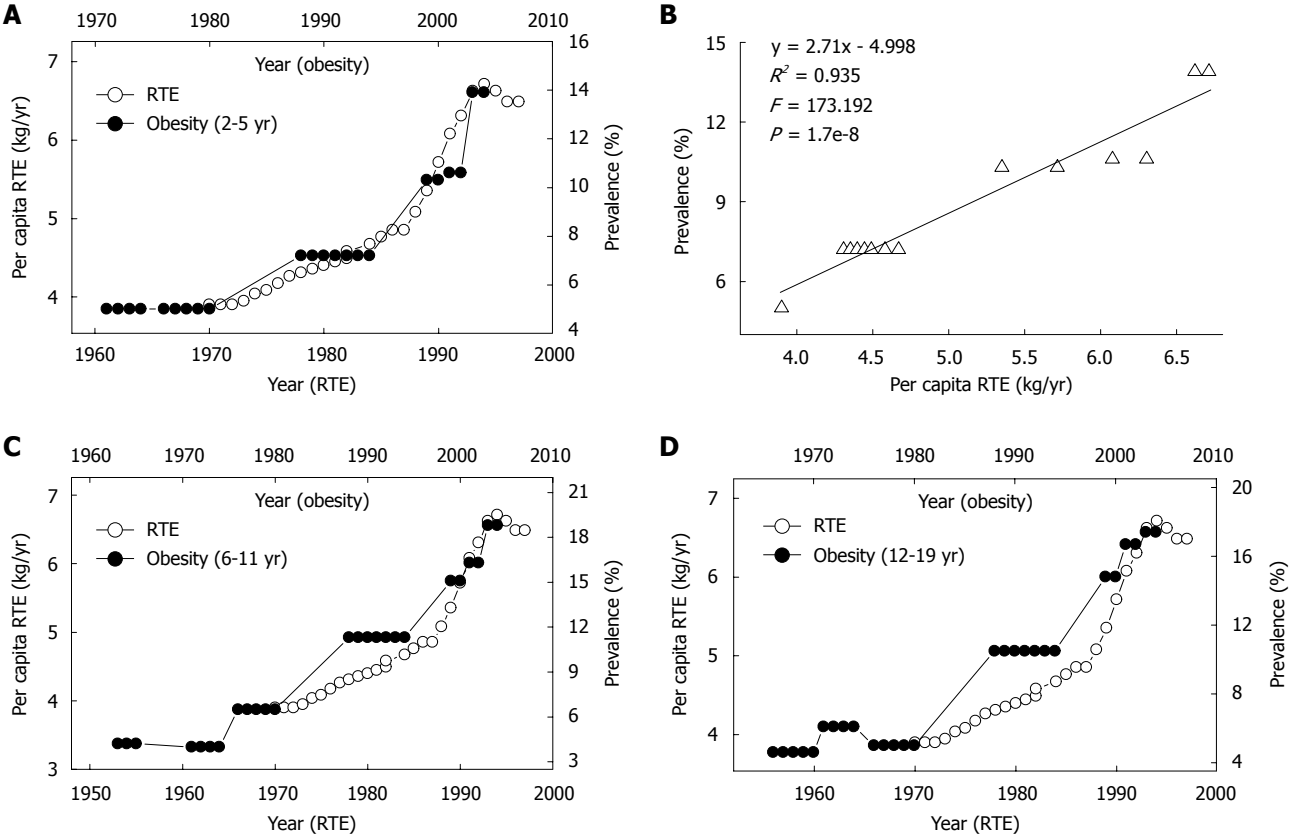


Figure 5 Relationship between per capita ready-to-eat cereal (RTE) consumption and prevalence of obesity in US children. A: Trends in US per capita RTE consumption in 1970-1997 (Ref. 32) and in the prevalence of obesity in the 2-5 years age group; B: The linear regression plot of the obesity prevalence in 1980-2004 against RET consumption in 1970-1994 using the data in A; C and D: A similar relationship between the RTE consumption and the obesity prevalence in the 6-11 years and 12-19 years age groups.

significant lag-correlations between the grain contribution to niacin and the obesity prevalence of US children aged 2-5 years (R^2 = 0.726, P = 4.8e-7), 6-11 years (R^2 = 0.898, P = 6.8e-13) and 12-19 years (Figure 4F).

Association between RTE consumption and the obesity prevalence in US children

ERT, a popular food item for many Americans, especially children, is the most common vehicle for niacin-fortification in the US. The continued upward trend of niacin since the mid-1970s has been primarily due to the increase in the fortification standards of RTE in 1974 and the greater use of enriched grain products[8]. As shown in Figure 5, the yearly per capita consumption of RTE has rapidly increased since 1970, and the obesity prevalence in the US children of all age groups increased in parallel with the increase in the RTE consumption with a 10-year lag. There were significant correlations between the RTE consumption in 1970-1994 and the obesity prevalence in US children aged 2-5 years (Figure 5B), 6-11 years (R^2 = 0.933, P = 2.1e-8) and 12-19 years (R^2 = 0.913, P = 1.0e-7) in 1980-2004.

DISCUSSION

The major findings of this study are: (1) nicotinamide overload may induce a biphasic effect on glucose metabolism, characterized by insulin resistance followed by hypoglycemia; and (2) there is a significant association between the US per capita niacin consumption and the obesity prevalence in US children and adolescents. These findings may help explain both the development of obesity and the increased prevalence of obesity.

Insulin resistance is a key feature of obesity[22]. Increasing evidence has indicated that systemic oxidative stress, characterized by elevation of plasma ROS, is an important trigger of insulin resistance[33]. In agreement with this suggestion, this study found that the plasma insulin level increased in parallel with the rise of plasma H_2O_2, the major mediator of oxidative stress, while decline in the H_2O_2 level led to hypoglycemia, which suggested that the insulin sensitivity increased. It seems that the oxidative stress plays a critical role in nicotinamide-induced insulin resistance. The present findings are in agreement with the hypothesis that niacin-induced increase in β-cell secretory capacity is the result of pancreatic islet adaptation to niacin-induced insulin resistance[25,26].

It is generally accepted that obesity results from excess energy intake and physical inactivity. A recent hypothesis suggests that factors favoring a trend toward hypoglycemia might induce excess energy intake and overweight[34]. Interestingly, the present study demonstrated that nicotinamide overload induced a biphasic response: insulin resistance in the early phase characterized by more insulin

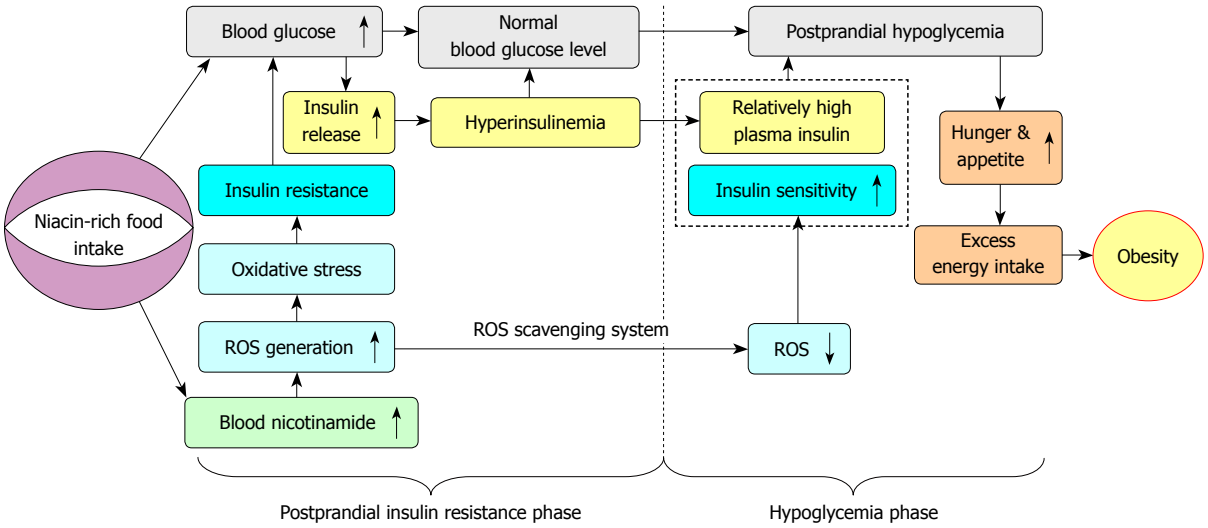


Figure 6 Proposed model of the role of niacin overload in obesity development. Excess nicotinamide metabolism may induce a biphasic effect: postprandial hyperinsulinemia followed by postprandial hypoglycemia, in which reactive oxygen species (ROS) generation and scavenging may play a central role.

release due to the enhanced ROS production, and hypoglycemia in the late phase due to the different clearance rates of plasma ROS and insulin. The biphasic response may underlie the increased appetite in obesity: high nicotinamide diet may produce more ROS and decrease insulin sensitivity, which leads to more insulin release. Then, with the relative rapid fall of plasma ROS and the re-increase in insulin sensitivity, the relative high insulin level may lead to hypoglycemia, which may induce hunger, eating behavior change, and subsequent excess energy intake (Figure 6). As such, it is not difficult to imagine that long-term nicotinamide overload-induced insulin resistance may eventually lead to β-cell failure. From this point of view, it seems that long-term excess nicotinamide intake may be a primary cause of obesity and type 2 diabetes. Moreover, our previous study demonstrated that sweat is an effective way to eliminate excess nicotinamide from the body[27]. Thus, physical activity not only increases energy expenditure but also decreases insulin resistance by facilitating the elimination of excess nicotinamide through sweating, which may help explain why sweat-inducing activities are effective in preventing obesity and type 2 diabetes. It should be pointed that excess niacin-induced oxidative stress and abnormal glucose metabolism is expected to involve other blood glucose-controlling hormones, such as glucagon and glucagon-like peptides. Further studies are warranted to examine the effect of excess niacin intake on these hormones.

Since niacin overload may be involved in the development of obesity, determining whether there is niacin overload in the modern diet may be helpful for understanding the global prevalence of obesity. Generally, dietary niacin comes mainly from the following two main sources: niacin-rich foods and niacin fortified foods. In term of the development of obesity and diabetes, the known high-risk foods are those rich in niacin, such as meat[35,36]. Most importantly, niacin fortified-grain products have been the most significant source of niacin. As shown in Figure 1, the daily US per capita niacin consumption has been kept a rising trend since the implementation of mandatory niacin-fortification, and has been two times higher than the RDA in the early 2000s. The amount of the daily per capita niacin consumption from grains and animal flesh was 3.7 and 6.8 mg, respectively, before the introduction of mandatory niacin-fortification in the 1930s, and has increased to 14.8 and 11.8 mg, respectively, in 2000. The percentage of grain contribution to dietary niacin has been increased four times since the implementation of niacin fortification. It is obvious that niacin-fortification may be mainly responsible for niacin overload, and may play a role in the increased prevalence of obesity in the US. To test this hypothesis, we examined the relationship between US per capita niacin consumption and the prevalence of obesity in US children and adolescents. Indeed, the present results showed that the obesity prevalence in the US children of all age groups increased in parallel with the increase in the per capita niacin consumption and the increase in the grain contribution to niacin, with a 10-year lag. RTE is a popular food item for many Americans, especially children. The update of fortification standards in the US in 1974 has led to a significant increase in niacin content in RTE[8]. The present study also revealed that there was a significant lag-correlation between the obesity prevalence in US children and the RTE consumption. Since the late 1990s, the US per capita niacin consumption has remained relatively stable (around 32-33 mg/d per capita)[9]. According to the regression equations shown in Figure 3, the obesity prevalence in the US children of all age groups should have been near its peak now. Indeed, the most recent NHANES data showed that there was no significant change in obesity prevalence between 2003-2004 and 2005-2006 in US children[37].

Dietary factors play a key role in the development and prevalence of obesity and diabetes[12,16,35,36]. The increasing global prevalence of obesity and diabetes implies that there may be a common change in diet composition worldwide. It should be pointed out that one of the common and major global changes in diet composition in the

last few decades is the significant increase in the content of dietary vitamins due to the spread of food fortification worldwide. The global prevalence of obesity and diabetes has spread in much the similar way as that of niacin-fortification, in contrast, those countries that had not introduced niacin-fortification or that prohibited niacin fortification before the early 1990s, such as Norway[38,39], have a low prevalence of obesity and diabetes in the 1990s, compared with the niacin-fortified countries such as the US and Canada. Thus, the possibility cannot be ruled out that the rapid global increase in the prevalence of obesity in the past three decades may be, at least in part, a man-made event due to niacin fortification.

Obesity is strongly associated with non-alcoholic fatty liver disease (NAFLD), a common disease characterized by an increase in intrahepatic triglyceride content with or without steatohepatitis[40]. As early as in 1964, Rikans *et al*[41] found that excess niacin in high fat diets can induce fatty liver in rats. Moreover, numerous case reports have shown that excess niacin can induce liver injury[6]. Thus, there is the possibility that excess niacin intake may play a role in the development of NAFLD. Investigating excess niacin metabolism and its toxic effects may be of help in gaining insight into the development of not only obesity but also NAFLD.

In summary, the present study demonstrates for the first time that nicotinamide overload-induced biphasic response in glucose metabolism may play a role in the development of obesity, and suggests that the high prevalence of obesity in US children and adolescents may involve a long-term niacin overload largely due to the grain fortification with niacin. It seems that the long-term safety of niacin fortification needs to be carefully evaluated.

COMMENTS

Background

Niacin, a potent stimulator of appetite, may induce insulin resistance at high doses. Global prevalence of obesity which is characterized by increased appetite and insulin resistance has occurred following the spread of grain fortification with niacin worldwide. However, how niacin stimulates appetite and whether excess dietary niacin plays a role in the obesity epidemic are not known.

Research frontiers

Obesity is directly related to diet and physical activity. Therefore, exploring the dietary risk factors for obesity and understanding the underlying mechanism of increased appetite and insulin resistance in obesity are important issues in the prevention and treatment of overweight and obesity.

Innovations and breakthroughs

The present study demonstrated for the first time that excess nicotinamide, when co-administered with glucose, induces biphasic response: insulin resistance in the early phase characterized by more insulin release due to the enhanced reactive oxygen species (ROS) production, and hypoglycemia in the late phase due to the different clearance rates of plasma ROS and insulin. The excess niacin-induced biphasic response may play a role in the development of obesity. This study also revealed for the first time that the obesity prevalence among US children and adolescents increased in parallel with the increase of the per capita niacin consumption with a 10-year lag, in which niacin fortification-induced sharp increase in niacin contents in grain products may play a major role. The present findings may help explain why there has been a sudden sharp increase in the obesity prevalence among US children and adolescents starting around the early 1980s, i.e. about 10 years after updating the niacin fortification standards in 1974.

Applications

Reducing niacin intake and facilitating niacin elimination through sweat-inducing physical activity may be a key factor in the prevention and treatment of obesity.

Peer review

Li *et al* studied in a small (pilot) study the effects of niacin overload on glucose metabolism in relation with a study performed in a large cohort, and this paper would be of interest.

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BRIEF ARTICLE

Quality of life after curative liver resection: A single center analysis

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Abstract

AIM: To evaluate quality of life (QoL) after curative liver resection and identify variables associated with decreased QoL.

METHODS: From October 2001 to July 2004, 323 patients underwent liver resection. At 3-36 mo after discharge, 188 patients were disease free. QoL was assessed using the Short Form (SF)-12 Health Survey with mental and physical component scales (SF-12 MCS and PCS), supplemented with generic questions concerning pain and liver-specific items.

RESULTS: Sixty-eight percent (128/188) returned the questionnaire, which was completed in 75% (96/128) of cases. Median SF-12 PCS and MCS were 46.7 (interquartile range: 34.2-53.9) and 54.1 (42.8-58.2). Fifty percent were pain free with a median symptom score of 1.75 (1.38-2.13). PCS was higher after major hepatectomy [57% (55/96)] compared to minor resection (P = 0.0049), which represented an improved QoL. QoL was not affected by sex but by age compared to the general German population. MCS was higher after liver surgery for metastatic disease [55.9 (47.5-58.8)] compared to primary carcinoma [49.6 (36.5-55.1)] and benign disease [49.2 (37.7-56.3)] (P = 0.0317). There was no correlation between length of postoperative period and QoL. Pain, deficiencies in everyday life and a high symptom score significantly decreased MCS and PCS.

CONCLUSION: Most patients were only marginally affected even after major liver resection; however, minor complications were associated with decreased SF-12 MCS and PCS and need careful attention.

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Key words: Quality of life; Liver resection

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INTRODUCTION

Over the past few decades, the multimodal concept of

quality of life (QoL) has been appreciated in many areas of medical practice[1]. Social, physical and mental factors contribute to QoL[2,3]. QoL assessment has proven to be a valuable parameter for patients and surgeons and may be helpful in determining the optimal treatment. As an outcome parameter, QoL is considered as important as disease-free and overall survival[4]. For many different procedures in surgery, the effect on QoL has been assessed, including liver resection for primary and secondary cancer, organ transplantation and gastrectomy[5-13].

In various benign and malignant liver diseases, resection is a common procedure with curative intention. While major and minor hepatectomy are both safe procedures, little is known about postoperative QoL in these patients[14-16]. Erim *et al*[17,18] have investigated QoL of live donors who have undergone liver resection for living related liver transplantation. Anxiety and depression increased in live donors, and improved 3 mo after surgery. Furthermore, Kaneko *et al*[19] have published a study on laparoscopic *vs* open liver resection in patients with hepatocellular carcinoma. QoL improved earlier after laparoscopic surgery. Their finding is similar to other studies that have compared various laparoscopic with open surgical procedures[19,20]. Recently, it has been published that QoL returns to baseline after liver resection for malignancies in most cases[21,22]. Martin *et al*[21] have shown in a prospective study of 36 patients that QoL returns to baseline at about 3 mo after liver resection for cancer.

Thus, the present study was designed to assess postoperative QoL of patients who underwent curative liver resection. Curative resection was defined as the absence of recurrent disease at the time of assessment. QoL was compared with that of the general German population, with a special focus on demographics (e.g. age and sex) and medical history (e.g. indication, type of resection, complications and impairment).

Although several studies on QoL after liver resection have been performed, to the best of our knowledge, this is the largest study on QoL of patients who have undergone curative liver resection[19,21,23,24]. In our opinion, this study's value is the exclusion of any bias caused by the palliative situation of the study population. It seems plausible that patients in a palliative situation might be impaired in their physical and especially mental QoL scores. Therefore, we decided to exclude any patients with signs of recurrence or active disease.

MATERIALS AND METHODS

Study population

Patients who underwent curative partial hepatectomy from October 2001 to July 2004 were recruited for this single-center, observational study on QoL after liver resection. While all patients underwent liver resection with curative intention, curative treatment in the context of this study was defined as the absence of recurrent disease at the time of assessment. The study protocol was approved by the ethical committee and all patients provided informed consent.

A total of 323 patients underwent partial hepatectomy in our hospital from October 2001 to July 2004. One hundred and eighty-eight patients were disease-free at the time of assessment and were eligible to participate in the study, and questionnaires were mailed to these patients. A return rate of 68% (128 patients) for the questionnaires, of which in 75% were filled out completely, further decreased the final study population to 96 patients. Thus, the final population consisted of almost 50% of the eligible study population.

Measures

QoL was assessed by the Short-Form (SF)-12 Health Survey, which is the abbreviated version of the widely used generic profile SF-36[25,26]. Both questionnaires have been validated for numerous populations and quantify the overall physical and mental aspects of QoL[27-34]. The SF-12 provides a subset of 12 items from which two summary measures of physical and mental health status (SF-12 PCS and SF-12 MCS) can be derived from subscales for physical function, physical role, pain, general health, vitality, social function, emotional role, and mental health with high validity, reliability and sensitivity[35].

The demographic and postoperative data, and results of the SF-12 Health Survey, a well-established QoL questionnaire, were analyzed, together with a pain assessment on a 0-10 scale and relevant liver-specific items.

Additional illness-specific items were used to assess liver-specific issues, for example, fever, dyspepsia, heartburn, lack of appetite, nausea, vomiting, night sweat and exhaustion. Patients indicated on a scale of 1-5 (1, never; 2, rarely; 3, sometimes; 4, often; 5, very often) the frequency of the individual symptom postoperatively. The calculated mean was used as a symptom score that ranged from 1 to 5. Impairment in everyday activities was measured on a scale of 1-5 (1, none; 2, light; 3, moderate; 4, heavy; and 5, strongest) and patient autonomy was measured as a variable indicating "independent" and "help needed". Pain was assessed using a 0-10 scale.

Surgery

As described by Belghiti *et al*[36], major hepatectomy was defined as resection of three or more segments and minor hepatic resection as resection of two or fewer segments. Thus, our patients were divided into a major and minor hepatectomy group.

Complications

Postoperative complications were stratified into surgical (e.g. bile leak or biloma, pneumothorax, wound infection, liver abscess, bleeding, and surgical dehiscence) and medical (e.g. pleural effusion, renal failure, hepatic failure, pneumonia, cardiac insufficiency, and cholangitis), and were assessed from patient records. Complications were defined as described elsewhere[16].

Study procedures

QoL was assessed at least 3 mo after discharge from hospital (range: 3-36 mo). Medical data were collected from

follow-up examinations as proof of patient eligibility (e.g. no signs of recurrence). In patients with malignant diseases, staging was performed according to the guidelines of the Association of the Scientific Medical Societies in Germany. Only patients without signs of recurrence were eligible for the study. Subsequently, questionnaires were sent to the patients by mail. Furthermore, patients were contacted by telephone to increase the return rate of questionnaires. Clinical data such as indication for liver resection, type of liver resection, medical and surgical postoperative complications were collected and documented in a database in a prospective manner.

Statistical analysis

SAS software (version 9.1; SAS Institute, Cary, NC, USA) was used for statistical analysis. The quantitative parameters SF-12 PCS, SF-12 MCS, pain, symptom score, and age are presented as median with interquartile range. Mann-Whitney *U* test and Kruskal-Wallis one-way analysis of variance were chosen to compare the quantitative parameters between subgroups of patients. Patients were stratified for age, sex, type of resection and indication for surgery. The relationships between the quantitative parameters were analysed using Spearman's rank correlation coefficient *r* and its corresponding *P* value. Two-sided *P* values were always calculated; $P < 0.05$ was considered statistically significant, and $P < 0.001$ was considered highly significant.

RESULTS

Study population

One hundred and eighty-eight patients underwent partial hepatectomy for benign and malignant hepatic lesions and were recurrence-free at the time of assessment. While 128 patients (68%) returned their questionnaire, it was fully completed in only 96 cases (52% of recurrence-free patients), of which 50% were male. Thus the final study population comprised 96 patients (Table 1). No significant difference could be found in demographics, indications and type of resection between patients with fully and partial filled questionnaires.

Indication for liver resection

The diagnoses are listed in Table 1; almost 21% of patients resected had benign lesions and > 79% had malignant liver tumors. Of the latter, > 72% were metastatic. More than 69% of metastases had spread from a colorectal tumor.

Surgical procedures

The procedures performed are listed in Table 1. There were 55 (57%) major and 41 minor hepatic resections. Patients underwent hemi-hepatectomy or extended hemi-hepatectomy in 45% and > 6% of the cases, respectively. Furthermore, six patients (6%) underwent resection of ⩾ 3 segments other than (extended) hemi-hepatectomy. Minor resection was performed in 43% of patients. These patients underwent resection of one segment (24%), or segmental resection that consisted of *en bloc* resection of two segments or resection of two discontinuous segments (20%).

Table 1 Demographics, indications and type of resection

	n = 96	100%
Median age (yr)	63.4	IQR: 54.5-70.5
Male/female	48/48	50/50
Primary malignant	21	21.9
Hepatocellular carcinoma	12	12.5
Cholangiocellular carcinoma	8	8.3
Gallbladder carcinoma	1	1.0
Metastatic	55	57.3
Colorectal	38	39.6
Other	17	17.7
Benign	20	20.8
Adenoma	4	4.2
Focal nodular hyperplasia	6	6.3
Cysts	1	1.0
Echinococcus	3	3.1
Haemangioma	2	2.1
Other	4	4.2
Major hepatectomy	55	57.3
Right hemi-hepatectomy	35	36.5
Extended right hemi-hepatectomy	3	3.1
Left hemi-hepatectomy	8	8.3
Extended left hemi-hepatectomy	3	3.1
Segmentectomy ($n > 2$)	6	6.3
Minor hepatectomy	41	42.7
Segmentectomy ($n = 1$)	23	24.0
Segmentectomy ($n = 2$)	18	18.8
Concomitant extrahepatic resection	10	10.4

Patients were stratified for demographic data, indications and type of resection. Patients suffering from benign, primary malignant and metastatic liver diseases were included. IQR: Interquartile range.

Morbidity

At least one of both medical and surgical postoperative complications occurred in about 30 (31%) patients (Table 2). Pleural effusion was the most frequent medical complication and occurred in about 9% of all cases. The most frequent surgical complication was bile leakage or biloma, which occurred in 6% of all cases (Table 2). Type of surgery (i.e. major or minor hepatectomy) was not significantly associated with frequency of surgical and medical complications.

QoL and symptom scores

Table 3 shows the SF-12 PCS and SF-12 MCS median scores stratified for demographic items, type of resection, indication and overall morbidity.

The age-dependent distribution of physical and mental SF-12 scores is shown in Figure 1 for patients *vs* the population norm. Patients younger than 40-51 years who underwent liver resection showed significantly lower SF-12 PCS (Figure 1A) and SF-12 MCS (Figure 1B) scores compared to their age-matched normal group ($P < 0.05$).

The mental QoL score was significantly higher ($P < 0.05$) in patients who underwent liver resection for

Table 2 Morbidity and postoperative complications

Complication	Total		Major hepatectomy		Minor hepatectomy	
	n = 96	100%	*n* = 55	100%	*n* = 41	100%
Total morbidity	30	31.2	20	36.4	10	24.4
Surgical complications	19	19.8	14	25.5	5	12.2
Bile leak/biloma	6	6.3	5	9.1	1	2.4
Pneumothorax	3	3.1	2	3.6	1	2.4
Wound infection	2	2.1	2	3.6	-	-
Abscess (liver)	1	1.0	1	1.8	-	-
Bleeding	1	1.0	1	1.8	-	-
Surgical dehiscence	1	1.0	1	1.8	-	-
Other	5	5.2	2	3.6	3	7.3
Medical complications	19	19.8	11	20.0	8	19.5
Pleural effusion	9	9.4	7	12.7	2	4.9
Renal failure	3	3.1	2	3.6	1	2.4
Hepatic failure	2	2.1	2	3.6	-	-
Pneumonia	2	2.1	-	-	2	4.9
Cardiac insufficiency	1	1.0	-	-	1	2.4
Cholangitis	1	1.0	1	1.8	-	-
Other	3	3.1	3	5.5	-	-
Revision laparotomy	3	3.1	2	3.6	1	2.4

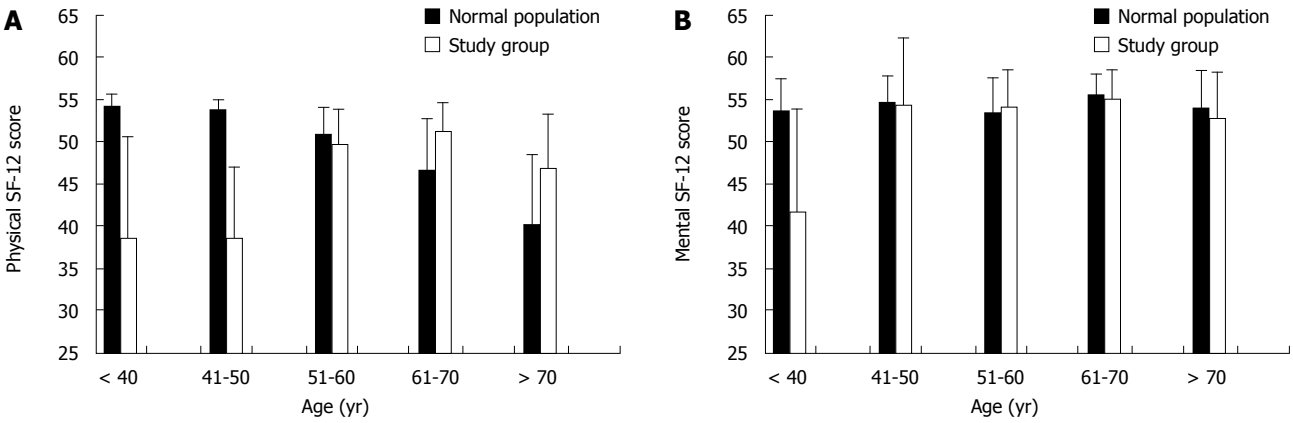


Figure 1 SF-12 scores *vs* general population. A: Physical SF-12 score. Although younger patients experienced lower physical scores than did the general population, the difference between the groups decreased; B: Mental SF-12 score. Older patients experienced the same levels of mental QoL as younger patients. The younger the patients were, the more significant was the impact of surgery on their scores.

metastases, with almost 56 (48-59) compared to patients with primary liver malignancies or benign liver disease with scores of about 49 (38-56) and 50 (37-55), respectively. Physical QoL varied according to the type of liver resection. While patients who underwent major resection reached a physical score of 52 (39-54), values after minor resection were about 41 (32-51) ($P < 0.005$). Sex, age and postoperative morbidity did not have a significant impact on mental or physical QoL.

Postoperative symptoms rarely occurred (Figure 2). The median value of symptom scores was 1.75 (1.38-2.13).

Subjective impairment after surgery correlated with lower summary score ($P < 0.001$) (Table 4). Patients who were not able to manage their living alone without the help of others had lower scores for both SF-12 PCS ($P < 0.05$) and SF-12 MCS ($P < 0.001$) (Table 4). The occurrence of medical problems considered to be minor was associated with decreased QoL scores (Table 4). Patients with postoperative wound infections showed significantly lower values for both SF-12 PCS ($P < 0.05$) and SF-12 MCS ($P < 0.05$) compared to patients without this complication and population norm.

High symptoms scores, pain and deficiencies had significant negative correlations to physical and mental SF-12 scores. Furthermore, high symptom scores correlated with high levels of pain and deficiencies in day-to-day life experienced by patients. Pain and scores of SF-12 PCS had a significant negative correlation ($r = -0.65$, $P < 0.0001$), while high degrees of deficiencies in the patients' daily routine correlated with low QoL values ($r = -0.59$ and -0.51 for PCS and MCS, respectively, $P < 0.0001$). Furthermore, high symptom scores were associated with pain and the feeling of being handicapped in life.

DISCUSSION

In mail surveys, non-responder bias has to be kept in mind. Our study provided valid data for 68% of our

Table 3 Median SF-12 scores

	n	Physical score		Mental score	
		Median	IQR	Median	IQR
Total	96	46.7	34.2-53.9	54.1	42.8-58.2
Sex					
Male	48	50.9	33.2-54.0	55.0	44.8-58.9
Female	48	44.6	36.3-53.4	52.9	40.9-57.8
Age (yr)					
< 41	10	38.5	33.4-50.4	41.8	32.0-54.1
41-50	7	38.6	35.4-46.9	54.4	37.1-62.4
51-60	16	47.4	33.3-54.9	54.6	43.6-59.8
61-70	38	49.8	36.0-54.2	55.3	43.1-58.8
> 70	25	50.8	37.4-53.8	53.4	45.0-57.9
Indication[a]					
Benign	20	43.5	33.4-54.9	49.2	37.7-56.3
Prim. malignant	22	41.6	32.9-53.1	49.6	36.5-55.1
Metastatic	54	50.6	39.0-54.2	55.9	47.5-58.8
Type of resection[b]					
Major	55	52.0	39.0-54.4	54.8	43.7-58.5
Minor	41	41.3	31.6-51.0	52.6	38.6-57.9
Morbidity					
Yes	30	44.2	29.7-53.8	53.1	43.1-57.8
No	66	50.2	37.4-54.1	54.1	42.4-58.8

Mental and physical SF-12 scores were stratified for sex, age, indication, type of resection and morbidity. Higher SF-12 scores represent better QOL. *P* values physical/mental scores: [a]$P = 0.2966/0.0317$ (metastatic *vs* primary malignant); [b]$P = 0.0049/0.4176$ (major *vs* minor resection).

Table 4 Impaired autonomy and wound healing after resection, and median physical and mental scores

	n = 96	Physical score	Mental score
Impairment			
None	35	53.8	57.9
Slight	30	43.3	54.4
Moderate	20	43.3	45.4
Heavy	9	33.7	36.9
Strongest	2	21.8	41.6
Autonomy			
Independent	90	48.4	54.6
Help needed[a]	6	28.3	41.6
Wound healing deficiency			
Yes	10	33.0	43.0
No	85	46.9	54.8
Not stated[b]	1	25.8	55.1

Questions on impairments in everyday activities, autonomy and wound healing were included in the survey. [a]$P < 0.0008$ for physical SF-12 scores, $P < 0.0354$ for mental SF-12 scores; [b]$P = 0.0474$ for physical SF-12 scores, $P = 0.0062$ for mental SF-12 scores.

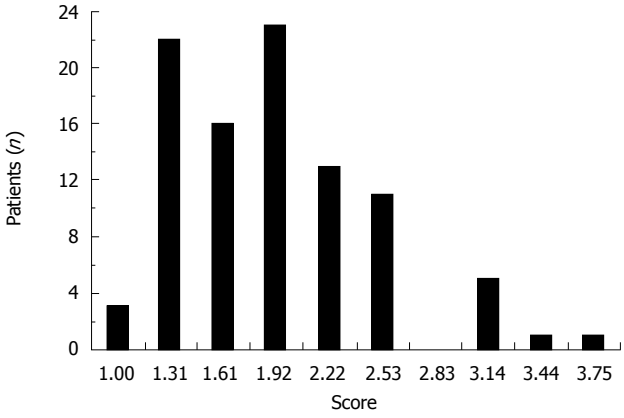


Figure 2 Distribution of liver-specific symptom score. Patients were asked about fever, dyspepsia, heartburn, lack of appetite, nausea, vomiting, night sweat and exhaustion. Patients were stratified for mean levels of symptom scores ranging from 1 to 5. The mean value of 1.82 ± 0.51 indicated that postoperative symptoms rarely occurred.

patients, and data for the missing 32% could only be generated by extrapolation. Different approaches exist for the extrapolation of data from mail surveys, which have been discussed previously[37-39]. Due to the relatively high response rate - compared to a mean response rate to mail surveys published in medical journals of 30%-60% - and since there was no systematic difference as far as demographic and medical characteristics were concerned, we assumed that our data were valid in the context in which they were investigated[38].

Postoperative QoL has been assessed for many medical and surgical treatments[40-43]. Most of these studies aimed not only at providing descriptive data, but also to help physicians and surgeons to choose optimal treatment[44]. Our results describe QoL after liver resection for patients who received curative treatment. In contrast to Martin *et al*[21], we included patients with all common types of indication for liver resection. Furthermore, we ensured that only patients who were disease free at least 3 mo after liver resection were included.

The present study found that patients younger than 50 years of age had lower QoL compared to their age-matched normal population group and to older patients. The impact of surgery on younger patients' QoL might

be stronger for several reasons. Besides medical reasons (i.e. comorbidity and different indications), it has to be taken into account that, in general populations, QoL (especially physical) is usually found to decline with age, and patients might have different expectations in different age groups. Patients older than 70 years of age reported a higher physical QoL than their age-matched controls.

Patients with liver metastases reported a higher physical as well as mental QoL than those who underwent resection for primary malignant or benign liver lesions (Table 3). We assumed that most patients who underwent surgery for metastatic disease already knew from the primary surgical intervention that physical wellbeing would return some time after surgery. Furthermore, we assumed that the fulfilled hope to be cured from the metastases of an already resected primary tumor might have a great impact on QoL[45].

Surprisingly, patients who underwent major liver resection reported a higher physical QoL compared to those who underwent minor resection (Table 3). Patients might compare their QoL to their recovery period, which may be prolonged and more complicated after extensive surgery, thus leading to a subjectively improved QoL.

Neither surgical nor medical complications were more frequent after major compared to minor hepatectomy (Table 2). Furthermore, postoperative complications that are considered to be minor medical problems, such as impaired wound healing, were found to interfere with QoL (Table 4). In all patients, the symptom score, pain and deficiencies in daily routine correlated significantly with QoL.

Data presented here show that 3-36 mo after discharge from hospital, mental and physical scores tended to be close to those of the general population, as long as QoL-decreasing factors (e.g. pain, or impaired wound healing) were absent. Furthermore, we were also able to identify important determinants of QoL.

QoL is always subject to individual judgement and compared by the patients to their own experience. Thus, elderly patients, who generally experience decreased physical QoL in comparison to younger patients, might find the effect of surgery less invasive. While this is clearly speculative, this might be an explanation for the higher physical QoL scores after surgery in elderly patients.

Moreover, individual judgement of QoL offers an explanation for the counter-intuitive finding of higher QoL scores after major hepatectomy in comparison to minor resection. Patients undergoing major resection most likely experience a higher impact of the more invasive surgery. Thus, after recovery, they might experience a higher change in their physical scores.

In contemporary surgery, QoL is considered as important as disease-free survival and morbidity. Thus, we investigated important factors that potentially determine QoL in patients undergoing curative hepatic resection. This study clearly demonstrates that even after a short time following liver resection, the vast majority of patients score equal or even higher in SF-12 compared to that of the population norm.

However, there is a small number of patients whose QoL might be affected by pain, impaired wound healing and subjectively perceived deficiencies in their daily routine following liver resection. This implies that appropriate pain and wound management is needed and coping with deficiencies in daily routine needs to be addressed postoperatively.

Furthermore, clinicians should be aware that QoL might be appraised differently depending upon age and underlying disease, in order to discuss the expectations of the surgical procedure.

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COMMENTS

Background

Quality of life (QoL) has been appreciated in many areas of medical practice and has proven to be helpful in determining the optimal treatment. As an outcome parameter, QoL is considered as important as disease-free and overall survival. Although major and minor hepatectomy are both safe procedures, little is known about their postoperative QoL. Thus, this study was designed to assess the postoperative QoL of patients who underwent curative liver resection and to compare their QoL to that of the general German population, with a special focus on demographics and medical history.

Research frontiers

Although several studies on QoL after liver resection have been performed, to the best knowledge of the authors of this article, this is the largest study on QoL of patients who underwent curative liver resection. In the authors' opinion, this study's value is the exclusion of any bias caused by the palliative situation of the study population. It seems plausible that patients in a palliative situation might be impaired in their physical and especially mental QoL scores. Therefore, the authors decided to exclude any patients with signs of recurrence or active disease.

Innovations and breakthroughs

In contrast to other studies, the authors eliminated the palliative bias and thus were able to evaluate the effect of surgery itself on QoL. While interpretation of our findings needs to be done with due care and, as in other studies on this subject, is speculative, they were able to identify contributors to QoL, and more importantly, did not identify a negative effect of curative hepatectomy on QoL.

Applications

The authors think that their findings are helpful in defining the optimal treatment for patients who require liver resection. Thus, their data justify the indication for surgery and can help surgeons to recommend major and minor hepatectomy to patients who need this type of surgery.

Terminology

QoL is a multimodal concept and can be measured with standardized questionnaires such as the Short-Form Health Survey (SF). Both versions (SF-12 and SF-36) have been validated and provide information on both physical and mental QoL.

Peer review

This is a large study of QoL in 323 patients undergoing liver resection for various indications. QoL was assessed using standard verification protocols.

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BRIEF ARTICLE

Narrow-band imaging endoscopy to assess mucosal angiogenesis in inflammatory bowel disease: A pilot study

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Abstract

AIM: To investigate whether narrow band imaging (NBI) is a useful tool for the *in vivo* detection of angiogenesis in inflammatory bowel disease (IBD) patients.

METHODS: Conventional and NBI colonoscopy was performed in 14 patients with colonic inflammation (8 ulcerative colitis and 6 Crohn's disease). Biopsy samples were taken and CD31 expression was assayed immunohistochemically; microvascular density was assessed by vessel count.

RESULTS: In areas that were endoscopically normal but positive on NBI, there was a significant ($P < 0.05$) increase in angiogenesis (12 ± 1 vessels/field *vs* 18 ± 2 vessels/field) compared with areas negative on NBI. In addition, in areas that were inflamed on white light endoscopy and positive on NBI, there was a significant ($P < 0.01$) increase in vessel density (24 ± 7 vessels/field) compared with NBI-negative areas.

CONCLUSION: NBI may allow *in vivo* imaging of intestinal angiogenesis in IBD patients.

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Key words: Narrow-band imaging; Angiogenesis; Inflammatory bowel disease; Crohn's disease; Ulcerative colitis

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INTRODUCTION

Angiogenesis plays a crucial role in neoplastic and non-neoplastic chronic inflammatory disorders[1-4], including the inflammatory bowel diseases (IBD), Crohn's disease (CD) and ulcerative colitis (UC)[4,5]. Potent angiogenic activity has been demonstrated in histological specimens from the mucosa of both UC and CD patients, as assessed by CD31 staining[6]. Several reports have shown that blockade of angiogenesis in preclinical models of IBD is a promising new therapeutic approach[7,8]. Narrow band imaging (NBI) is a new endoscopic technology that highlights mucosal surface structures and microcapillaries. Optical filters achieve sequential green and blue illumination, narrowing the bandwidth of spectral transmittance, and thus obtaining tissue illumination at selected narrow

wavelength bands[9,10] to achieve the greatest contrast between vascular structures and the surrounding mucosa.

The diagnostic accuracy of NBI in detecting colorectal neoplasia in patients with or without concomitant UC is superior to conventional colonoscopy and equivalent to that of chromoendoscopy[11,12]. A recent meta-analysis concluded that NBI is accurate, with high diagnostic precision for *in vivo* diagnosis of neoplasia across a range of organs (colon, esophagus, duodenal ampulla and lung)[13].

Very recently, NBI has been proposed as a tool to assess the grade of inflammation in patients with inactive or mildly active UC[14]. In the preliminary study described herein, we investigated whether NBI colonoscopy could be a useful tool to detect *in vivo* angiogenesis in IBD patients with colonic inflammation.

MATERIALS AND METHODS

This was an open study involving patients with a diagnosis of IBD referred to our Gastrointestinal Endoscopy Unit for follow-up colonoscopy. A total of 14 patients were included (8 UC and 6 colonic CD). The extent of the disease was determined by previous colonoscopy. At the time of enrollment in the study, 3 (3/8) UC patients presented with inactive disease (Mayo score = 0) while 5 (5/8) had active disease (2 patients Mayo score = 1, 2 patients Mayo score = 2 and 1 patient Mayo score = 1); 3 (3/6) patients with CD presented with inactive disease and 3 (3/6) had active disease. For CD patients endoscopic activity was assessed by Crohn's Disease Endoscopic Index of Severity (CDEIS). After obtaining informed consent from all patients, white light colonoscopy and NBI (Olympus Medical System, Tokyo, Japan) examinations were performed.

For the white light colonoscopy, the vascular pattern was defined as normal if it did not show any irregularities, or as distorted if the pattern was tortuous. When the vascular pattern intensity was visualized with NBI, we were able to distinguish 2 different mucosal patterns: a stronger (blacker) capillary vascular pattern (NBI+), and a milder or regular capillary vascular pattern (NBI-). For this reason, in our study the vascular pattern could be classified into 4 categories: normal (with white light colonoscopy) and NBI-; distorted (with white light colonoscopy) and NBI-; normal (with white light colonoscopy) and NBI+; distorted (with white light colonoscopy) and NBI+.

For each patient, after determining the vascular pattern by NBI, biopsy specimens were obtained from 5 areas that were normal with conventional endoscopy and NBI-, 5 areas that were normal with conventional endoscopy but NBI+, and 5 areas that were endoscopically inflamed and NBI+. CD31 staining was performed by immunohistochemistry, and microvascular density was assessed by vessel count in colonic biopsies. The pathologist was blinded to the subjects.

Statistical analysis

The parametric data are expressed as the mean ± SD and non parametric data as percent. Fischer's exact probability test and the χ^2 test were used to evaluate statistical differences. A *P*-value less than 0.05 was considered statistically significant.

RESULTS

For each of the 14 patients enrolled in the study, the mucosal vascular pattern was assessed by both conventional and NBI colonoscopy.

Mucosal areas normal with white light colonoscopy and NBI-

In the uninflamed mucosa of patients with IBD, classified as normal with white light colonoscopy and NBI-, the NBI pattern was similar to that in the mucosa from healthy control individuals. At the same time, the vascularization pattern and the microvessel density, as revealed by CD31 staining, were similar in the specimens from the uninflamed, NBI- mucosa from patients with IBD and from healthy controls. No differences were found between UC and CD patients.

Comparison of NBI+ and NBI- areas that were normal with white light colonoscopy

Compared to areas that were endoscopically normal under white light colonoscopy and NBI-, endoscopically normal but NBI+ areas displayed a significant ($P < 0.05$) increase in angiogenesis (12 ± 1 vessels/field *vs* 18 ± 2 vessels/field) (Figure 1A and B). The importance of our findings lies in the evidence that in patients with normal white light colonoscopy, areas positive on NBI showed an increased leukocyte infiltrate and a significantly increased microvessel density ($P < 0.05$) as assessed by histological analysis (Figure 1A-C). As revealed by staining for CD31, the mean microvessel diameter in IBD was 0.1 mm, a size histologically compatible with the diameter of a dot observed on the NBI image. No differences were found between UC and CD patients (not shown).

Areas inflamed on white light colonoscopy and NBI+

Lastly, in areas from IBD patients that were inflamed under white light endoscopy and were NBI+, a significant ($P < 0.01$) increase in vessel density (24 ± 7 vessels/field) was found compared with endoscopically normal, NBI- areas (Figure 2A and B), a finding compatible with a high degree of microscopical inflammation and immune-driven angiogenesis. No differences were found according to Mayo score in vessel density (not shown). No differences were found between UC and CD patients (not shown).

DISCUSSION

Angiogenesis is an integral component of non-neoplastic chronic inflammatory disorders, such as IBD[15,16]. Microscopic imaging is the approach that has thus far proven most valid for quantification of vasculature in normal and pathological tissue[17-19]. NBI has been successfully used to visualize angiogenesis and thereby to detect cancerous areas in the colon. Because of the improved mucosal contrast provided, NBI may improve the detection of colon polyps compared with standard

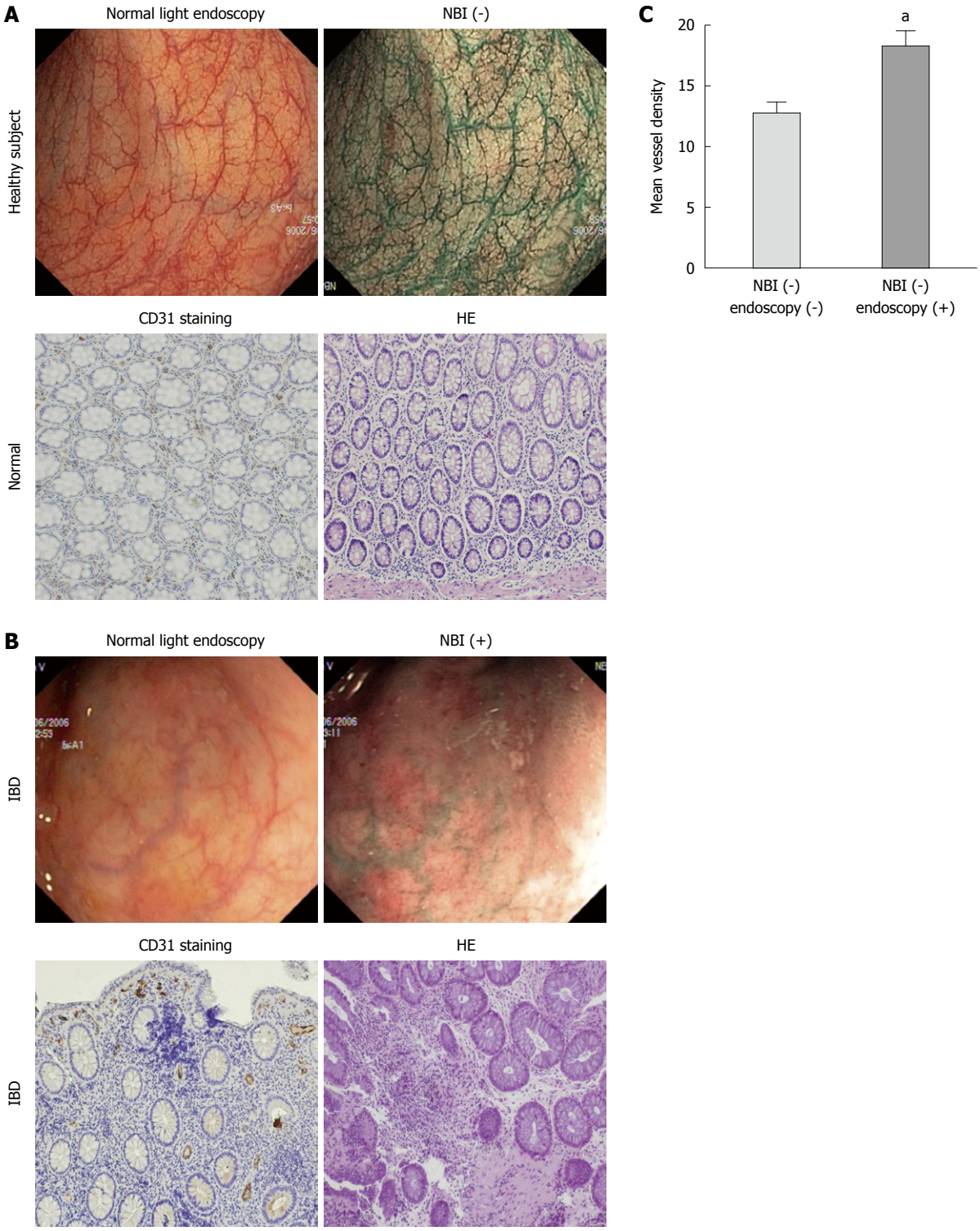


Figure 1 Colonic mucosa of healthy individuals and uninflamed but narrow-band imaging (NBI)+ regions from patients with inflammatory bowel disease (IBD), visualized using white light colonoscopy and NBI endoscopy. The microvasculature of histologically normal control and IBD uninflamed colonic mucosa was immunohistochemically stained for CD31 and von Willebrand/factor Ⅷ. There was increased vascularization in uninflamed IBD mucosa that presented an aberrant NBI+ pattern, in the NBI+ areas compared with controls. A: Healthy subjects. The vascular pattern was normal on both conventional colonoscopy and NBI, which was confirmed by immunohistochemical staining; B: Areas of the IBD mucosa that were not inflamed on conventional colonoscopy and showed an aberrant NBI+ pattern. Immunohistochemical staining confirmed an increased vascularization in these areas compared with NBI- areas; C: Computerized morphometric analysis of the microvasculature in control and IBD uninflamed mucosa that was NBI+. After immunohistochemical staining, sections were analyzed for the total number of vessels/field (microvascular density). [a]A statistically significant difference was found between sections from areas of uninflamed IBD colonic mucosa that were NBI+ and from controls.

white light colonoscopy. NBI has also been widely used to detect dysplasia in patients with long-standing UC, achieving good results in terms of diagnostic accuracy and detection of polyps, although if did not show any statistically significant differences compared with standard white light colonoscopy[19,20].

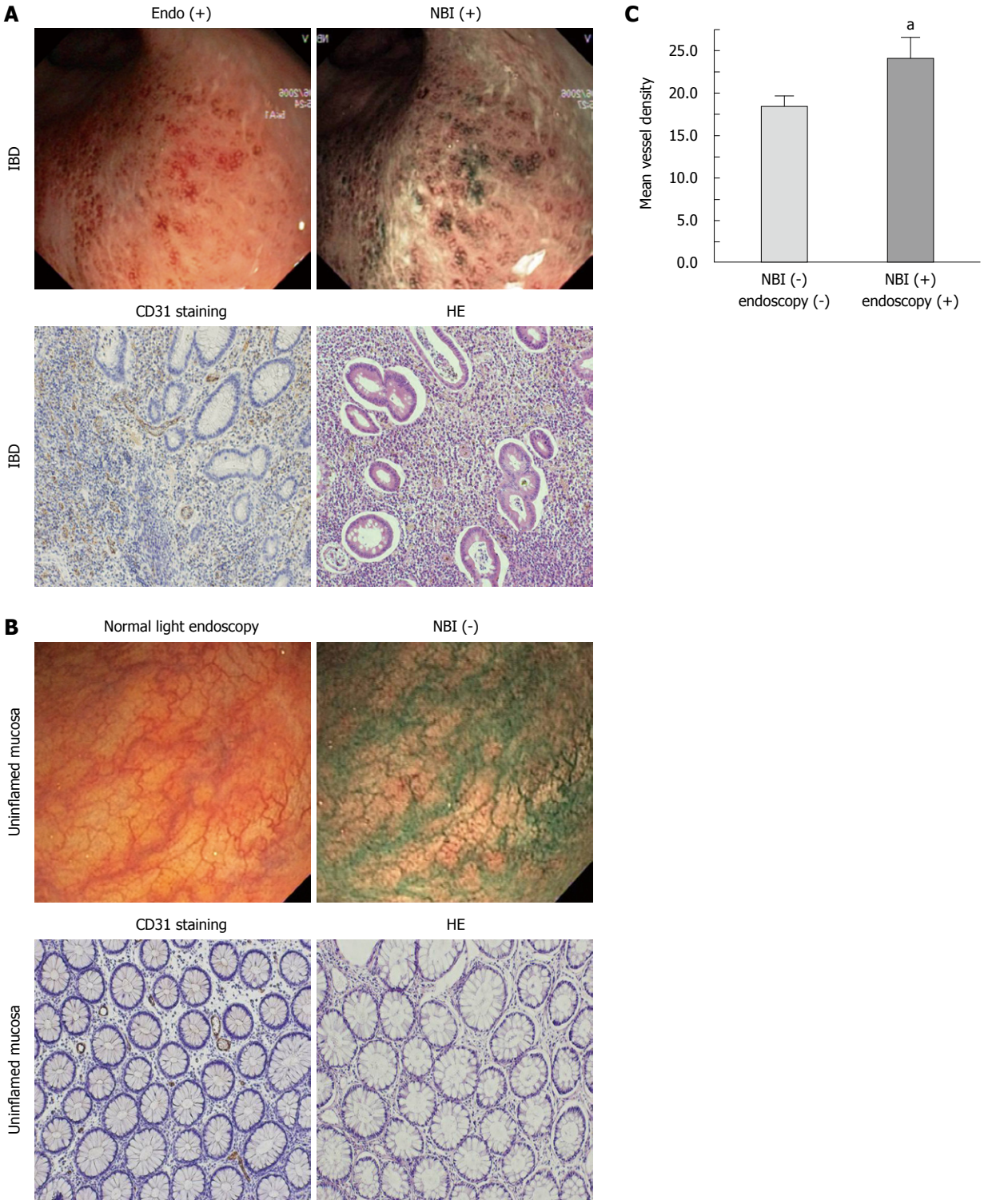


Figure 2 Inflamed colonic mucosa of IBD patients visualized using white light colonoscopy and NBI. The microvasculature of IBD inflamed colonic mucosa was immunohistochemically stained for CD31 and von Willebrand/factor Ⅷ. A: Areas of the IBD mucosa that were inflamed on white light colonoscopy showing an aberrant NBI+ pattern. There was increased vascularization in the inflamed IBD mucosa in areas where there was an aberrant NBI+ pattern, which was confirmed by immunohistochemical staining; B: Areas of the IBD mucosa that were not inflamed on conventional colonoscopy and showed an aberrant NBI+ pattern. Immunohistochemical staining confirmed an increased vascularization in these areas compared with NBI- areas; C: Computerized morphometric analysis of the microvasculature in the IBD inflamed mucosa. After immunohistochemical staining, sections were analyzed for the total number of vessels/field (microvascular density). [a]A statistically significant difference was found between sections from areas of inflamed IBD colonic mucosa that were NBI+ and areas of uninflamed IBD mucosa that were normal under white light colonoscopy but were NBI+ .

In a recently published study, NBI was proposed as a tool to assess the grade of inflammation in patients with inactive or mildly active UC[12]. In our study, we found that NBI could also be used to visualize areas of abnormal microvascular changes, not observed at white light colonoscopy. A statistically significant correlation exists between NBI pattern positivity (both in inflamed and normal areas at conventional endoscopic examination) and microves-

sel density, as confirmed by CD31 staining in histological specimens from the same areas. In patients who were normal under standard colonoscopy, there was increased leukocyte infiltration and microvessel density in NBI+ areas, as assessed by histological analysis.

Blockade of angiogenesis could be beneficial in patients with chronic inflammation and some drugs that have demonstrated efficacy for the treatment of IBD, such as tumor necrosis factor-α inhibitors, have potent antiangiogenic activity. Our preliminary findings suggest that NBI could be a novel tool for the *in vivo* assessment of mucosal angiogenesis. However since our study is still a preliminary study because of the limited number of patients, a larger study should be performed to define the exact role of NBI in IBD patients, and the correlation of mucosal angiogenesis with endoscopic activity.

Endoscopic imaging and monitoring of angiogenesis has the potential to be a valuable biomarker in monitoring the grade of intestinal inflammation *in vivo*, monitoring response to and optimization of available treatments, and finally in evaluating the response to new agents for the treatment of IBD in randomized clinical trials.

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COMMENTS

Background

Angiogenesis plays a crucial role in neoplastic and non-neoplastic chronic inflammatory disorders, including inflammatory bowel diseases (IBD).

Research frontiers

Narrow-band imaging (NBI) is a new endoscopic technology that highlights mucosal surface structures and microcapillaries. A recent meta-analysis concluded that NBI is accurate, with high diagnostic precision for *in vivo* diagnosis of neoplasia across a range of organs (colon, esophagus, duodenal ampulla and lung).

Innovations and breakthroughs

Very recently, NBI has been proposed as a tool to assess the grade of inflammation in patients with inactive or mildly active ulcerative colitis. In this preliminary study, the authors investigated whether NBI colonoscopy could be a useful tool to detect *in vivo* angiogenesis in IBD patients with colonic inflammation.

Applications

Several reports have shown that blockade of angiogenesis in preclinical models of IBD is a promising new therapeutic approach. Visualize angiogenesis *in vivo* may represent the first step for such a therapeutic approach.

Terminology

Angiogenesis: the process of new capillary formation from pre-existing vasculature in adult tissues; Narrow-band imaging: a new endoscopic technology that highlights mucosal surface structures and microcapillaries.

Peer review

The authors report that NBI may be a novel modality for imaging of intestinal angiogenesis in IBD. The results provide sufficient evidence to draw scientific conclusions. The statistical data reflect the results and are adequate for a clinical study. The discussion is well organized, and valuable conclusions are provided.

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BRIEF ARTICLE

Disagreement between symptom-reflux association analysis parameters in pediatric gastroesophageal reflux disease investigation

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Abstract

AIM: To assess the agreement within 3 commonly used symptom-reflux association analysis (SAA) parameters investigating gastroesophageal reflux disease (GERD) in infants.

METHODS: Twenty three infants with suspected GERD were included in this study. Symptom index (SI), Symptom sensitivity index (SSI) and symptom association probability (SAP) related to cough and irritability were calculated after 24 h combined pH/multiple intraluminal impedance (MII) monitoring. Through defined cut-off values, SI, SSI and SAP values are differentiated in normal and abnormal, whereas abnormal values point towards gastroesophageal reflux (GER) as the origin of symptoms. We analyzed the correlation and the concordance of the diagnostic classification of these 3 SAA parameters.

RESULTS: Evaluating the GER-irritability association, SI, SSI and SAP showed non-identical classification of normal and abnormal cases in 39.2% of the infants. When irritability was taken as a symptom, there was only a poor inter-parameter association between SI and SSI, and between SI and SAP (Kendall's tau b = 0.37, $P < 0.05$; Kendall's tau b = 0.36, $P < 0.05$, respectively). Evaluating the GER-cough association, SI, SSI and SAP showed non-identical classification of normal and abnormal cases in 52.2% of the patients. When cough was taken as a symptom, only SI and SSI showed a poor inter-parameter association (Kendall's tau b = 0.33, $P < 0.05$).

CONCLUSION: In infants investigated for suspected GERD with pH/MII-monitoring, SI, SSI and SAP showed a poor inter-parameter association and important disagreements in diagnostic classification. These limitations must be taken into consideration when interpreting the results of SAA in infants.

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Key words: Gastroesophageal reflux disease; Infant; Symptom-reflux association analysis; Intraluminal impedance monitoring; pH

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INTRODUCTION

Gastroesophageal reflux disease (GERD) remains one of the most common diagnoses made by pediatric gastroenterologists. By definition, gastroesophageal reflux (GER) is the involuntary passage of gastric contents into the esophagus or oropharynx, which is a physiological process that appears in every individual particularly after meals[1]. When GER causes troublesome symptoms and/or complications, it is referred to as GERD[2]. Clinical manifestations in infants include among others regurgitation, vomiting, irritability and cough[1]. A main problem in the diagnosis of GERD is the association of symptoms with GER, especially the non-specific ones. While endoscopy can detect GER complications such as mucosal inflammation, symptom-reflux association analysis (SAA) is the only available method that can adequately identify GER as the cause of short lived symptoms[3].

SAA aims to show and quantify a temporal relation between symptoms and GER. With qualification, a significant temporal relation between symptoms and GER, thus an abnormal result of the SAA parameter, may suggest GERD. Three parameters are often used to address this temporal relationship: Symptom index (SI), symptom sensitivity index (SSI) and symptom association probability (SAP)[3-6]. A pre-condition required for SAA is a technical measure that identifies GER episodes in the individual esophagus. Two methods can be used: pH-monitoring and a combined pH/multiple intraluminal impedance (MII) monitoring.

In infants, GERD occurs frequently during the first months of life. During this period GER is often non-acid, because the frequent milk intake acts as a potent buffer of gastric acidity. pH-monitoring alone has the disadvantage of only detecting acid GER. The newer MII technique to measure GER allows the additional detection of non-acid GER and thus more GER events. This is the main difference from pH-measurement alone. It is likely that a combined pH/MII measurement will replace single pH-measurement in the future[4,7,8].

Defined cut-off values of the SAA parameters help to decide whether symptom episodes which occurred during GER monitoring are related to GER or not. Despite routine clinical use in pediatrics[6], SAA parameters have never been validated in children or in infants. The problem is the lack of an objective and reliable gold standard to which these parameters can be compared, as well as the ethical difficulty posed by obtaining data from healthy children. The correlation of these 3 parameters as well as the concordance of abnormal results within these parameters in infants is unknown. Therefore, a comparison between studies using different SAA parameters is not possible. Additional questions arise as to whether only one SAA parameter could be a substitute for the others or how to interpret different results of the various SAA parameters measured in one individual.

Condino *et al*[5] reported that in infants hospitalized for evaluation of GER, fussiness/pain and cough were the most frequent symptoms and were found to be the most frequent symptoms related to GER detected by combined pH/MII monitoring. We assessed SI, SSI and SAP for irritability and cough in a group of infants who underwent combined pH/MII monitoring for suspected GERD. In order to evaluate the agreement between these 3 parameters we analyzed the correlation and the concordance between them in normal and abnormal classification.

MATERIALS AND METHODS

In this retrospective study, combined pH/MII tracings of consecutively investigated infants performed between October 2006 and January 2009 were reviewed. All infants included had irritability and cough among other GER-like symptoms and underwent 24 h combined pH/MII monitoring for suspicion of GERD. Patients were clinically evaluated prior to combined pH/MII analysis. Various investigations such as barium swallow, esophagogastroduodenoscopy and laboratory analysis were performed based on history and clinical signs. A barium swallow was performed in infants with suspected anatomic upper gastrointestinal anomalies. Esophagogastroduodenoscopy was performed in the case of GERD resistant to therapy and to detect eosinophilic esophagitis. Diagnosis of esophagitis was based on histology of esophageal biopsies[9]. Therapy was prescribed by the referring physicians or by one of the authors (P.B.). Therapy was not discontinued during the entire observation period.

Twenty four h combined pH/MII measurement was performed using hardware and software by Sandhill Technologies (Sandhill Scientific Inc., Highlands Ranch, CO, USA). A single-use catheter (ConforTEC® pH-MII probe ZIN-BS 45) with a diameter of 2.13 mm was placed transnasally. This probe consisted of six 1.5 cm impedance recording segments and one pH-electrode, located within the distal impedance segment. The middle of each impedance segment was located at 0, 1.5, 3, 4.5, 6 and 7.5 cm above the pH-electrode. The initial positioning of the catheter was estimated using the following formula: (0.156 × height in cm) + 6.88 cm = length of pH-electrode from nose to distal esophagus[10]. Proper positioning of each catheter was confirmed by X-ray. The pH-electrode was placed 2 vertebral bodies above the diaphragm with a tolerance margin of 5 mm. The probe was connected to the portable recording device. An external reference electrode was attached posteriorly to the patient's chest.

The studies were performed on an inpatient basis. The caregivers (nurse and parents) were properly instructed by the attending physician on how to register the irritability and cough events on the portable recording device. We defined the term "irritability" as crying or whining. A child was irritable either if it cried or if it showed behavior of being unwell, expressed as making a grimace on the verge of crying. The latter was the perception of the parents. The pH/MII tracings were evaluated using the BioVIEW analysis software (Sandhill Scientific, Inc.) and each study was manually reviewed by a pediatric gastroenterologist. Patients with less than 20 h of recording were excluded from the study. A (liquid) GER episode detected by im-

pedance was defined as a retrograde drop in impedance by more than 50% of baseline in the distal 2 channels[11]. Gas only episodes were not included in the analysis. A symptom was considered related to a GER event if it occurred within a 5 min window from the GER event[12,13].

The SI was defined as the number of GER-related symptoms divided by the total number of symptoms, reflecting the percentage of symptoms related to GER episodes[12]. The SSI was defined as the number of GER-related symptoms divided by the total number of GER episodes, reflecting the percentage of GER associated with symptoms[14]. SAP was defined as the likelihood that the patients' symptoms were related to GER based on a statistical analysis (cross tabulation) of a contingency table consisting of 4 possible combinations of GER and symptoms. The SAP was calculated as $(1\text{-}P) \times 100\%$, with the *P*-value calculated using Fisher's exact test[15]. The SAP calculation was provided in the software package of the pH/MII monitoring device. Based on the literature, SI values of $\geqslant 50\%$, SSI values of $\geqslant 10\%$ and SAP values of $\geqslant 95\%$ were considered abnormal[3,5,6].

Statistical analysis

Statistical analysis was performed with SAS 9.2 (The SAS Institute, Cary, NC, USA). Continuous variables are presented as median and interquartile range (IQR) or, if an approximately normal distribution was assumed, as mean ± SD. *P*-values < 0.05 were considered significant. Concordance between the different SAA methods was assessed by Kendall's tau-b. Kendall's tau-b calculates a measure of concordance based on the number of concordant and discordant pairs and uses a correction for tied pairs. It ranges between -1 and +1, where +1 is perfect concordance and -1 perfect discordance. A value of zero indicates the absence of any concordance. In the case of continuous variables, rankings were compared[16].

The study was approved by the local ethics committee of the University of Fribourg, Switzerland.

RESULTS

Patient characteristics

Twenty-three infants aged 2 wk to 11 mo (median 3 mo, IQR 2.8 mo) were included in this study. 19 patients were male. One patient was under ranitidine treatment, 4 patients were under sucralfate treatment, 3 patients were under omeprazole treatment and 7 patients had thickened feeding. Mean duration of the procedure was 22 h and 44 min ± 1 h 11 min. A previous barium meal was performed in 11 patients and showed a hiatus hernia in 1 case. Two patients had undergone esophagogastroduodenoscsopy and biopsies after combined pH/MII monitoring, which showed esophagitis in both.

A total of 1692 GER episodes were detected, 395 were acid (23%) and 1297 non-acid (77%). 421 irritability episodes were reported, 165 (39%) were related to GER. There were 155 cough episodes, 73 (47%) related to GER. Table 1 summarizes the characteristics of the registered GER and symptom episodes.

Table 1 Characteristics of the registered reflux and symptom episodes

	GER episodes	Irritability episodes	Cough episodes
Min	22	2	1
Max	141	82	19
Median	67	11	5
IQR	48	18	10
Sum	1692	421	155

GER: Gastroesophageal reflux; Min: Minimal number for a single patient; Max: Maximal number for a single patient; Median: Median value for all patients; IQR: Interquartile range.

Symptom-reflux association analysis

We investigated the GER-irritability association and found the following overall values: SI 25%, IQR 37.5%; SSI 4.5%, IQR 8.1%; and SAP 54%, IQR 78%. Additionally, we investigated the GER-cough association and found the following overall values: SI 50%, IQR 75%; SSI 3.6%, IQR 4.3%; and SAP 38.4% ± 43.3%. Abnormal results for the GER-irritability association were found for 8 patients in SI (34.8%), for 5 patients in SSI (21.7%), and 3 patients in SAP (13.0%). We found abnormal results for the GER-cough association in 13 patients for SI (56.5%), 2 patients for SSI (8.7%), and in 1 patient for SAP (4.3%). Evaluation of the diagnostic classification in normal and abnormal cases for the GER-irritability association showed 60.8% of the patients with identical classification, and 39.2% with non-identical classification. Evaluation of the diagnostic classification in normal and abnormal cases for the GER-cough association showed 47.8% of the patients with identical classification, and 52.2% with non-identical classification. Table 2 summarize the data obtained.

All SAA parameters for the GER-irritability association were positive in one infant. This infant showed a notable GER on barium swallow without an anatomic anomaly. One infant had 3 positive SAA parameters for the GER-cough association. This infant had chronic esophagitis on esophagogastroduodenoscopy.

Evaluation of the diagnostic classification between the GER-irritability SAA parameters showed the following values: SI and SSI Kendall's tau b = 0.2791, $P = 0.2153$; SI and SAP Kendall's tau b = 0.2593, $P = 0.2748$; SSI and SAP Kendall's tau b = 0.4219, $P = 0.1618$. Evaluation of the diagnostic classification between the GER-cough SAA parameters showed the following values: SI and SSI Kendall's tau b = 0.2707, $P = 0.1255$; SI and SAP Kendall's tau b = 0.1870, $P = 0.2987$; SSI and SAP Kendall's tau b = 0.6908, $P = 0.2847$.

We found a poor correlation between the parameters SI and SSI in both symptom categories, irritability and cough. We also found a poor correlation between SI and SAP for the irritability symptom category (Table 3).

DISCUSSION

We investigated the agreement of different SAA parameters in infants who underwent combined pH/MII moni-

Table 2 Categorization of SI, SSI and SAP

SI	SSI	SAP	Number of infants	Percent	Cumulative frequency	Cumulative percent
Irritability/reflux association						
Abnormal	Abnormal	Abnormal	1	4.35	1	4.35
Abnormal	Abnormal	Normal	2	8.70	3	13.04
Abnormal	Normal	Abnormal	1	4.35	4	17.39
Abnormal	Normal	Normal	4	17.39	8	34.78
Normal	Abnormal	Abnormal	1	4.35	9	39.13
Normal	Abnormal	Normal	1	4.35	10	43.48
Normal	Normal	Normal	13	56.52	23	100.00
Cough/reflux association						
Abnormal	Abnormal	Abnormal	1	4.35	1	4.35
Abnormal	Abnormal	Normal	1	4.35	2	8.70
Abnormal	Normal	Normal	11	47.83	13	56.52
Normal	Normal	Normal	10	43.48	23	100.00

SI: Symptom index, abnormal if SI ⩾ 50%; SSI: Symptom sensitivity index, abnormal if SSI ⩾ 10%; SAP: Symptom association probability, abnormal if SAP ⩾ 95%.

Table 3 Correlations between SI, SSI and SAP for the irritability/reflux association and the cough/reflux association expressed in Kendall's tau b

	Irritability			Cough		
	SI	SSI	SAP	SI	SSI	SAP
SI	-	0.37102 (P = 0.0147)	0.36011 (P = 0.0225)	-	0.32725 (P = 0.0362)	0.20959 (P = 0.2117)
SSI	0.37102 (P = 0.0147)	-	0.23623 (P = 0.1297)	0.32725 (P = 0.0362)	-	0.29632 (P = 0.0701)
SAP	0.36011 (P = 0.0225)	0.23623 (P = 0.1297)	-	0.20959 (P = 0.2117)	0.29632 (P = 0.0701)	-

toring for suspected GERD using unspecific symptoms such as irritability and cough. To the best of our knowledge this is the first study in children to compare the correlation and concordance of the diagnostic classification between SAA parameters. Taken together SI, SSI and SAP show important disagreements in the diagnostic classification of normal and abnormal symptom-GER association. Within the different SAA parameters a week correlation could be seen at most. This renders the interpretation of the SAA results and the diagnosis of GERD based on SAA parameters difficult as the detection of a pathologic symptom association depends on the SAA parameter chosen. Additionally, the comparison between different studies should be based on the same SAA parameters because our study shows that SI, SSI and SAP are not interchangeable.

Our study showed a higher frequency of non-acid GER (77%) compared to acid GER. This is comparable to the incidence of non-acid GER in infants found in the literature[17]. The high proportion of non-acid GER in our study could also be explained by the fact that some of our patients were examined under treatment. To leave the patients under treatment while presenting GER-like symptoms reflects a clinical situation common in pediatric gastroenterology, as pediatric gastroenterologists are currently increasingly asked to evaluate a patient on proton pump inhibitor treatment after an unsuccessful medical trial. Condino *et al*[5] found no relation between category of GER (acid or non-acid) and the association between GER and the symptoms irritability and cough. In that study, 49.8% of fussiness and pain episodes (comparable to irritability) were related to GER, whereas in our study only 39% of the irritability symptoms were related to GER. In addition, it was reported that 33.5% of cough episodes were related to GER, whereas our study shows that 47% of cough episodes were related to GER.

While our findings are interesting, obviously the limitations of our study must be pointed out. As with many other studies investigating the role of MII in children, the sample size is rather small[18]. Evaluation of the diagnostic classification did not show significant concordance. This is probably due to true discordance, but could also be due to a too weak study power. Furthermore, it must be noted that this study predominantly consists of male infants which reduces the possibility of generalizing the findings to female infants.

The question arises as to which of the three SAA parameters is the best for the diagnosis of GERD. To date the only study to evaluate the diagnostic accuracy of SI, SSI and SAP is a work by Taghavi *et al*[19] on an adult study group. That study validated the SAA parameters obtained on 24 h pH-monitoring against a PPI treatment-trial dividing the study group into responders and non-responders. Patients were given a high dose of omeprazole treatment and asked to complete a symptom score before and after the treatment. Those who showed an effect were considered to have GERD. SSI, with an adapted cut-off value of 1.3%, showed the highest positive and negative predictive values, followed by SAP. SI had the poorest performance

with absence of a meaningful cut-off value making it almost useless. Despite the routine clinical use of SAA parameters in pediatrics there is currently no validation study for combined pH/MII monitoring. The problem is the lack of an objective gold-standard test for the detection of GERD, independent from combined pH/MII monitoring. In an analogy to the work of Taghavi *et al*[19], we would need to perform a reference test in infants, with symptom scoring before and after treatment (e.g. proton pump inhibitors, prokinetics, surface agent, *etc*.) including combined pH/MII monitoring. This is ethically and methodologically challenging in infants.

In general use of SAA is hampered by numerous question marks concerning its accuracy. Firstly, it depends on a technique that correctly detects the GER events. It is likely that the introduction of combined pH/MII-monitoring adding the detection of non-acid GER events, compared to single pH-monitoring, makes SAA more accurate. A study of Rosen *et al*[6] showed that significantly more children had a positive SI using combined pH/MII-monitoring, than using pH-monitoring alone. A possible shortcoming of the 24 h-monitoring technique is the fact that the number of acid and non-acid GER events in MII varied significantly on 2 consecutive days in an adult study[20]. Additionally, investigating 48 h ambulatory pH-monitoring doubled the SI and SAP in adults with atypical GER symptoms compared to 24 h monitoring[21]. Furthermore, automatic MII analysis considers only a drop of impedance of 50% or more as a GER event; however, it is very likely that a drop of 49% could also be attributed to a GER event[4]. To date there are very few studies which evaluate the efficacy of automated analysis. A study by Roman *et al*[22] in adults however showed good agreement between visual analysis and the automated analysis used in our study.

Secondly, results of SAA may have some methodological weaknesses. It depends on the active participation of the observers, in our case nurse and parents[4], thus being impacted by the compliance and the variable work intensity on the ward. It has been shown that adults record only 39% of the cough episodes detected by simultaneous manometry[23]. The arbitrary choice of the time window influences the symptom association parameters. The 5 min time window used within this automatic analysis tool and in other studies may not be optimal for the evaluation of symptoms such as irritability and cough. In this context one has to keep in mind, that SAA detects timely association, which does not necessarily mean causal relation. Unspecific symptoms in infants such as irritability and cough can be evoked by a large number of factors. The temporal association of GER and an unspecific symptom can therefore be a coincidence. SI and SSI have cut-off values that were arbitrarily chosen. In addition, they do not take into account the total number of GER episodes and total number of symptoms, respectively. Therefore, the SI can be positive simply because of a high number of GER episodes, whereas the SSI can be falsely positive because of a high number of symptoms[18].

By showing poor inter-parameter association and important disagreements in diagnostic classification with the 3 established SAA parameters SI, SSI and SAP using combined pH/MII monitoring technique, we showed a common problem encountered with SAA, which is the difficulty of choosing the appropriate parameter to use. Our study cannot give an answer to this problem. Because of unanswered technological, methodological and validation questions and the limited concordance between the SAA parameters, we believe that at the moment, SAA based on combined pH/MII monitoring is a poorly reliable tool for the diagnosis of GERD. Diagnosis of GERD in infants with GERD-like symptoms therefore remains based on several elements such as the absence of more probable reasons for GERD-like symptoms, the positive response to treatment or the detection of GERD complications. However, combined pH/MII-monitoring can argue against GER as the origin of GERD-like symptoms, when showing absent or very low frequencies of GER episodes. Studies in infants are needed in order to obtain reference values and to adjust SI, SSI and SAP cut-off values for GERD, validated for the infant population.

COMMENTS

Background

Gastroesophageal reflux (GER), defined as passage of gastric contents into the esophagus is a normal process that occurs in healthy infants, children and adults. When GER causes troublesome symptoms and/or complications it is referred to as gastroesophageal reflux disease (GERD). During infancy GER is common and can manifest with specific symptoms as vomiting and non-specific symptoms as irritability and cough. Association of non-specific symptoms with GER is a main problem in the diagnosis of GERD.

Research frontiers

Combined pH/multiple intraluminal impedance (MII) detects GER episodes in the individual esophagus. The better the timely association of GER episodes and symptom episodes the more probable it is that GER is the origin of symptoms. Timely association of GER episodes and symptom episodes are expressed by symptom-reflux association analysis (SAA) parameters. Abnormal results of SAA parameters point towards GERD. Three SAA parameters are commonly used, symptom index (SI), symptom sensitivity index (SSI) and symptom association probability (SAP) but they were never validated for the diagnosis of GERD in the infant population. In this study the authors analyzed the agreement between SI, SSI and SAP.

Innovations and breakthroughs

This is the first study in infants to show an important disagreement between the 3 commonly used SAA parameters SI, SSI and SAP, which puts the accuracy of SAA into question. The study shows that the results of SI, SSI and SAP for the diagnosis of GERD often differ. In consequence, the diagnosis of GERD in infants cannot be based on a single SAA parameter as it remains unknown which SAA parameter is the most accurate for the diagnosis of GERD.

Applications

The limited agreement of these parameters must be taken into consideration when interpreting the results of SAA. The diagnosis of GERD should be based on a combination of pH/MII-monitoring, SAA results as well as on other factors such as clinical judgment, gastroscopy and follow-up under medical therapy. Validation studies to enhance the accuracy of SAA parameters and to answer the question which SAA parameter is the most accurate are needed.

Terminology

MII in combination with pH-measurement, a technique based on the fact that the passage of gastric content into the esophagus changes the impedance (electrical resistance) between esophageal segments, is more and more replacing the classic single pH-measurement as a diagnostic tool for GERD. A catheter with multiple impedance recording segments and one distal pH-electrode

connected to an exteriorly portable recording device is placed transnasally into the patient's esophagus in order to detect acid and nonacid GER events.

Peer review

This is an important topic and it has been little addressed in children.

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BRIEF ARTICLE

Alkaline phosphatase predicts relapse in chronic hepatitis C patients with end-of-treatment response

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Abstract

AIM: To investigate relapse predictors in chronic hepatitis C (CHC) patients with end-of-treatment response (ETR), after pegylated interferon-α (PegIFN-α) and ribavirin treatment.

METHODS: In a retrospective study we evaluated a spectrum of predictors of relapse after PegIFN-α and ribavirin treatment in 86 CHC patients with ETR. Viral loads were determined with real-time reverse transcription polymerase chain reaction. Hepatitis C virus genotyping was performed by sequencing analysis. Patients with genotype 1 were treated for 48 wk with 180 μg PegIFN-α2a or 1.5 μg/kg PegIFN-α2b once weekly plus ribavirin at a dosage of 1000 mg/d for those under 75 kg or 1200 mg/d for those over 75 kg. Patients with genotypes 2 and 3 were treated for 24 wk with 180 μg PegIFN-α2a or 1.5 μg/kg PegIFN-α2b once weekly plus ribavirin at a dosage of 800 mg/d.

RESULTS: In all ETR patients, binary logistic regression analysis identified absence of complete early virological response (cEVR) (OR 27.07, 95% CI: 3.09-237.26, $P < 0.005$), serum alkaline phosphatase (ALP) levels prior to therapy < 75 U/L (OR: 6.16, 95% CI: 2.1-18.03, $P < 0.001$) and body mass index > 26 kg/m^2 (OR: 8.27, 95% CI: 2.22-30.84, $P < 0.005$) as independent predictors of relapse. When cEVR patients were analyzed exclusively, ALP prior to therapy < 75 U/L remained the only predictor of relapse.

CONCLUSION: Lower levels of ALP prior to, during and after therapy seem to be associated with a higher risk of relapse in CHC patients with ETR.

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Key words: Alkaline phosphatase; Chronic hepatitis C; Pegylated interferon; Predictor; Relapse

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INTRODUCTION

Chronic hepatitis C (CHC) is a major public health problem due to late complications, such as liver failure and hepatocellular carcinoma[1]. The prevalence of CHC has been estimated to be 3% worldwide[2]. Pegylated interferon-α (PegIFN-α) with ribavirin is currently the

treatment of choice for CHC[3]. Treatment response depends on patient and viral factors, the viral genotype being the most important[4]. Several studies have defined further predictive factors for response, such as hepatitis C virus (HCV)-RNA serum level, stage of liver disease, body weight, age, sex and race[5-10]. After completion of treatment, HCV may become detectable again considerably reducing sustained virological response (SVR) rates even after achievement of end-of-treatment response (ETR). The relapse rate of genotype 1 seems to be higher than in other genotypes, being as high as 30%-40%[11]. The risk of relapse was also shown to relate to treatment duration, and several efforts have been undertaken to identify those patients who would benefit from extended treatment duration[12].

In this retrospective study we investigate in a cohort of ETR patients whether further predictors of relapse may exist after treatment with PegIFN-α and ribavirin.

MATERIALS AND METHODS

Patient population

We reviewed the records of all CHC patients with an ETR who started PegIFN-α and ribavirin treatment between April 2005 and August 2007 at our clinic. Exclusion criteria were age < 18 years; any bone or bowel diseases, chronic renal failure, use of medication that could cause an elevation of liver enzymes; biliary obstruction confirmed sonographically; hepatitis B virus or human immunodeficiency virus co-infection; daily ethanol ingestion greater than 20 g for women and 40 g for men; drug abuse; history of previous antiviral treatment. Finally, 86 patients (62 men and 24 women, mean age 42.5 ± 11.3 years, range 18-67 years) were included. Genotypes 1, 2 and 3 were present in 52 (60.5%), 9 (10.5%) and 25 (29%) patients, respectively. Patients with genotype 1 were treated for 48 wk with 180 μg PegIFN-α2a or 1.5 μg/kg PegIFN-α2b once weekly plus ribavirin at a dosage of 1000 mg/d for those under 75 kg or 1200 mg/d for those over 75 kg. Patients with genotypes 2 and 3 were treated for 24 wk with 180 μg PegIFN-α2a or 1.5 μg/kg PegIFN-α2b once weekly plus ribavirin at a dosage of 800 mg/d. Patients with extended treatment duration were not included in this study. A complete early virological response (cEVR) was considered present, when HCV-RNA by polymerase chain reaction (PCR) was negative 12 wk after initiation of therapy. Patients, who did not achieve a cEVR showed at least a partial early virological response (pEVR), defined as a 2-log-decline of HCV-RNA at 12 wk after the initiation of therapy compared to baseline viral load. ETR was defined as negative HCV-RNA at the end of treatment, and SVR was defined as persistent absence of HCV-RNA by PCR at 6 mo, thereafter.

Laboratory analysis

Alkaline phosphatase (ALP), alanine aminotransferase, aspartate aminotransferase and γ-glutamyl transferase were assessed by routine colorimetric assays on the Modular Clinical Analyzer (Roche, Vienna, Austria). Diagnostic ranges were 1-1200 U/L, 4-600 U/L, 4-800 U/L and 3-1200 U/L, respectively. Normal ranges of ALP were 40-129 U/L for males and 35-104 U/L for females. The precision of the ALP assay was > 99.3%.

Viral loads were determined with the Cobas TaqMan HCV assay (Quantification range 10 to 6.9×10^7 IU/mL; Roche). HCV genotyping was performed by sequencing with the TruGene™ HCV 5'NC Genotyping Kit (Siemens Medical Solutions Diagnostics, Bad Nauheim, Germany).

Liver biopsy was available in 33 patients. Stages of liver fibrosis and inflammatory activity were classified according to the Metavir score[7].

Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (version 10, SPSS Inc., Chicago, Illinois, USA). Values are expressed as mean ± SD or as median (range). Differences between groups were compared by Student's *t*-test or Mann-Whitney *U* test where appropriate. Fisher's exact test was used to assess possible differences in categorical variables between patients with SVR and relapse. Possible correlations between variables were analyzed using the Spearman rank test. Binary logistic regression analysis was performed to identify independent predictors of relapse. Significance tests were 2-sided, and *P*-values < 0.05 were considered significant.

RESULTS

The clinical characteristics of the ETR patients are summarized in Table 1. Relapse occurred in 26 patients (30.2%), 17 patients (65.4%) with genotype 1, three patients (11.5%) with genotype 2 and 6 patients (23.1%) with genotype 3. The frequency of patients with genotype 1 was not significantly different between the relapse and the SVR group (Table 1). Median ALP prior to therapy was significantly higher, whereas mean body mass index (BMI), stage of liver fibrosis and viral load prior to therapy were significantly lower in patients with SVR compared to those with relapse. The frequency of cEVR was significantly higher in the SVR group compared to the relapse group (Table 1).

In all ETR patients, binary logistic regression identified absence of cEVR (OR 27.07, 95% CI: 3.09-237.26, $P < 0.005$), ALP prior to therapy < 75 U/L (OR: 6.16, 95% CI: 2.1-18.03, $P < 0.001$) and BMI > 26 kg/m^2 (OR: 8.27, 95% CI: 2.22-30.84, $P < 0.005$) as independent predictors of relapse. When only cEVR patients were analyzed, ALP prior to therapy < 75 U/L (OR: 7.79, 95% CI: 1.96-30.98, $P < 0.005$) remained the only predictor of relapse.

Prior to therapy, all ALP levels in patients with relapse were within the normal range.

During PegIFN-α and ribavirin treatment, ALP levels showed variations: in patients with SVR there was a significant decline, whereas in patients with relapse there was a slight non-significant variation (Figure 1A). Similar results were obtained when patients with genotype 1 were analyzed, only (Figure 1B).

Table 1 Clinical and biochemical characteristics of the patients prior to therapy

	SVR (n = 60)	Relapse (n = 26)	P-value
Age (yr)	41.3 ± 11.3	45.4 ± 10.9	NS
Male/female	43/17	19/7	NS
Body mass index (kg/m^2)	24.9 ± 4.0	28.7 ± 5.5	< 0.010
Systolic blood pressure (mmHg)	119 ± 14	119 ± 12	NS
Diastolic blood pressure (mmHg)	73 ± 10	75 ± 10	NS
Genotype 1, n (%)	35 (58.3)	17 (65.4)	NS
HCV-RNA prior to therapy (IU/mL)	480 500 (2430-23 000 000)	806 000 (107 000-8 400 000)	< 0.050
PegIFN α2a/PegIFN α2b	32/28	14/12	NS
cEVR, n (%)	58 (96.7)	17 (65.4)	< 0.001
Histological fibrosis stage	1 (0-4)	3 (1-4)	< 0.050
Histological activity	1 (1-3)	2 (1-3)	NS
Aspartate aminotransferase (U/L)	40 (15-416)	47.5 (24-156)	NS
Alanine aminotransferase (U/L)	72.5 (17-560)	70 (32-214)	NS
γ-glutamyl transferase (U/L)	39 (3-693)	48 (11-183)	NS
Alkaline phosphatase (U/L)	84 (40-232)	67 (48-129)	< 0.005
Cholinesterase (kU/L)	7.22 ± 2.18	7.50 ± 2.37	NS
Lactate dehydrogenase (U/L)	190 ± 42	193 ± 34	NS
Total bilirubin (mg/dL)	0.77 ± 0.57	0.68 ± 0.24	NS
C-reactive protein (mg/dL)	0.2 (0-1.8)	0.1 (0-1.0)	NS
Fibrinogen (mg/dL)	256 ± 52	273 ± 54	NS

SVR: Sustained virological response; HCV: Hepatitis C virus; PegIFN: Pegylated interferon; cEVR: Complete early virological response; NS: Not significant.

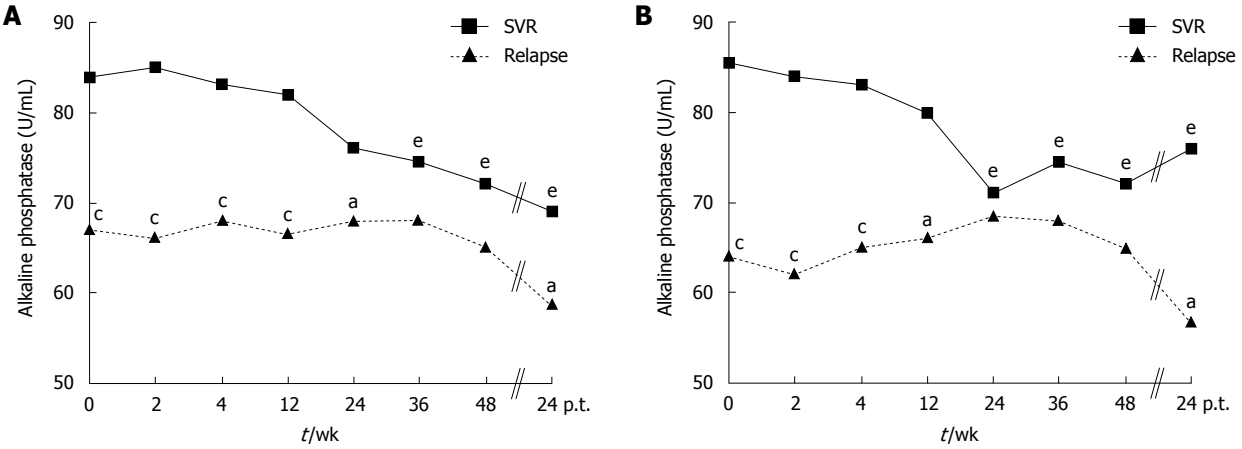


Figure 1 Time course of median serum levels of alkaline phosphatase (ALP) in patients with sustained virological response (SVR) and patients with relapse. A: All patients with genotypes 1-3 from week 0 to week 24. The 36 and 48 wk values exclusively represent genotype 1 patients (n = 52), as patients with genotypes 2 and 3 were only treated for 24 wk; B: Genotype 1 patients, only. $^aP < 0.05$ *vs* SVR; $^cP < 0.005$ *vs* SVR; $^eP < 0.05$ *vs* baseline. p.t.: Post treatment.

DISCUSSION

The results of our study indicate that serum levels of ALP are related to the risk of relapse in CHC patients with ETR after treatment with PegIFN-α and ribavirin.

Elevated levels of ALP are found in bone, bowel and bile duct diseases. As our study did not include patients with bone or bowel diseases, the observed significant difference in pre-treatment ALP between patients with relapse and those with SVR might be caused mainly by differing amounts of ALP from the liver or the bile ducts as the primary source of disease. Bile duct inflammation has been reported in up to 95% of HCV patients and was shown to correlate with serum ALP[13]. Since immune response and inflammation play key-roles in the elimination of HCV, the higher pre-treatment ALP levels in patients with SVR may possibly reflect a higher degree of inflammation with a more sustained response to therapy. However, the histological activity score in our patients failed to show this, as there was no significant difference between patients with SVR and relapse, which could be due to our limited sample size. The observed decline in ALP during PegIFN-α and ribavirin treatment may possibly be due to a decrease of intrahepatic inflammation, which initially could have been higher in those patients with SVR. PegIFN-α and ribavirin may also influence bone metabolism and levels of bone-derived ALP, however, other studies were unable to confirm these findings[14-16]. Any pathophysiological reasoning for the association of ALP levels and risk of relapse of CHC is speculative at present.

Our study is the first to focus on ETR-patients treated with PegIFN-α and ribavirin. Most patients in our study exhibited ALP levels within the normal range with

only 5% of patients with SVR exceeding the upper limit of the normal range. The limits of normal ranges are different in males and females, but gender distribution was almost equal in both patient groups.

In previous studies, genotype 1 was associated with increased relapse rates compared to the other genotypes[11]. In our study, the relapse rate in genotype 1 patients was not significantly higher probably due to the limited number of patients studied.

The strongest predictor of relapse in our study was absence of cEVR. Nearly all patients with SVR and almost two thirds of patients with relapse exhibited a cEVR. When cEVR is achieved, treatment duration is usually not extended. As prolongation of treatment has been shown to reduce relapse rates, ALP may possibly help to identify those patients, who would benefit from extended treatment duration[12].

Limitations of our study comprise its retrospective nature, limited sample size and lack of histological examinations in all patients. As ALP is elevated in a variety of diseases, we cannot exclude that some subclinical diseases with minor ALP elevations confounded our data. Despite those limitations, median ALP was higher in our SVR patients compared to those with relapse at all time points.

In conclusion, lower levels of ALP prior to, during and after therapy with PegIFN-α and ribavirin seem to be associated with a higher risk of relapse in patients with ETR. Further studies are required to clarify whether our observation could help to identify those patients who would benefit from extended therapy despite the presence of cEVR.

COMMENTS

Background

Chronic hepatitis C (CHC) is a major public health problem due to late complications, such as liver failure and hepatocellular carcinoma. Pegylated interferon-α (PegIFN-α) together with ribavirin is currently the treatment of choice for CHC. After achievement of end-of-treatment response (ETR) in CHC patients, the occurrence of relapse reduces sustained virological response (SVR) rates.

Research frontiers

Treatment response in CHC patients depends on patient and viral factors, the viral genotype being the most important. Several studies have defined further predictive factors for response, such as hepatitis C virus (HCV)-RNA serum level, stage of liver disease, body weight, age, sex and race. In this study the authors investigated in a cohort of ETR patients, whether further predictors of relapse may exist after treatment with PegIFN-α and ribavirin.

Innovations and breakthroughs

Previous studies investigated predictors of relapse in HCV infected individuals after PegIFN-α and ribavirin therapy. Those studies examined responding as well as non-responding patients, and predictive factors for response, such as HCV-RNA serum level, stage of liver disease, body weight, age, sex and race were identified. After completion of treatment, recurrence of the virus is still a major issue and a quest has been initiated to determine whether there may be predictors of relapse, in order to identify those patients who might benefit from extended treatment duration up front. The result of this study would suggest that alkaline phosphatase (ALP) might be such a predictor in ETR patients. This study is the first to focus on ETR patients, exclusively.

Applications

The authors suggest that apart from monitoring the already known predictors of relapse, ALP monitoring should be included in the work-up of HCV infected patients in preparation for anti-HCV treatment. Furthermore, ALP as an inexpensive clinical parameter should be monitored through-out the treatment period. Their results warrant larger studies to be conducted in a prospective fashion, in order to elucidate the clinical value of ALP monitoring in HCV patients undergoing treatment.

Peer review

It's a very interesting paper and I propose it to be published. Sure AP is not the only important tool to guarantee a SVR but can be useful to speak with patient's about this possibility.

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BRIEF ARTICLE

Increased levels of homocysteine in patients with ulcerative colitis

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Abstract

AIM: To investigate serum levels of homocysteine (Hcys) and the risk that altered levels carry for thrombosis development in ulcerative colitis (UC) patients.

METHODS: 55 UC patients and 45 healthy adults were included. Hcys, vitamin B12 and folic acid levels were measured in both groups. Clinical history and thromboembolic events were investigated.

RESULTS: The average Hcys level in the UC patients was 13.3 ± 1.93 μmmol/L (range 4.60-87) and was higher than the average Hcys level of the control group which was 11.2 ± 3.58 μmmol/L (range 4.00-20.8) ($P < 0.001$). Vitamin B12 and folic acid average values were also lower in the UC group ($P < 0.001$). When multivariate regression analysis was performed, it was seen that folic acid deficiency was the only risk factor for hyperhomocysteinemia. Frequencies of thromboembolic complications were not statistically significantly different in UC and control groups. When those with and without a thrombosis history in the UC group were compared according to Hcys levels, it was seen that there were no statistically significant differences. A negative linear relationship was found between folic acid levels and Hcys.

CONCLUSION: We could not find any correlations between Hcys levels and history of prior thromboembolic events.

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Key words: Ulcerative colitis; Homocysteine; Folate; Vitamin B12

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INTRODUCTION

Inflammatory bowel diseases (IBD) are chronic disorders of unknown etiology characterized by destructive inflammation of the gastrointestinal tract, acute exacerbations and remission periods. IBD is exemplified by two major groups of diseases. These include ulcerative colitis (UC) and Crohn's disease (CD). These two diseases are indistinguishable in 10%-15% of IBD patients. This is termed

Indeterminate Colitis (IC)[1]. Patients with IBD are at increased risk of experiencing thromboembolic complications. The incidence of arterial and venous thromboembolic diseases has been reported to be 1%-8% in UC and CD, however some autopsy studies have reported this incidence to be as high as 39%[2,3]. In the study of Bernestein *et al*[4], the risks of deep vein thrombosis and pulmonary emboli were found to be 3 times higher than that of the general population. In addition, venous thrombosis occurs earlier in IBD patients compared to the general population[5]. Thromboembolism is a multifactorial condition. Studies have indicated a state of hypercoagulability involving all the components of the coagulation system in patients with UC[6-8]. The exact etiology and pathogenesis of hypercoagulability have not been explained, however it is widely accepted that this condition is associated with acquired or genetic defects of the coagulation system and the procoagulant effect of proinflammatory cytokines[9-14]. This hypercoagulability increases the risk of thromboembolic events and has a role in the pathogenesis of UC *via* formation of microthrombi in the intestinal microcirculation[15,16].

Homocysteine (Hcys), which was first defined in 1932 by Butz and Vigneaud, is a sulphur-containing amino acid synthesized during transformation of methionine to cysteine in methionine metabolism[17]. Mild hyperhomocysteinemia occurs in about 5%-7% of the general population[18-24]. Increased Hcys levels are due to enzyme abnormalities in metabolic pathways or to nutritional deficits, including folate and vitamin B2, B6 and B12 deficiencies[25,26]. The exact relationship between hyperhomocysteinemia and induction of thrombosis is unknown, however, a state of hypercoagulability resulting from endothelial dysfunction has been blamed[26,27].

Vitamin B12 and folic acid deficiencies due to malnutrition, malabsorption and antifolate medications including methotrexate and sulphasalazine are quite common in IBD. The deficiency of these key nutrients leads to increased Hcys levels in IBD. Several recent studies have reported increased Hcys levels in IBD[28-33]. The objective of this study was to determine levels of homocysteine, folic acid and vitamin B12 in patients with UC; to compare these data with those of a healthy control group; and to investigate the relationship between homocysteine levels and disease activation, thromboembolic complications, localization of disease, and levels of folic acid and vitamin B12.

MATERIALS AND METHODS

Subjects

A total of 55 UC patients [38 males, 17 females; mean age (± SD) 47.4 ± 13.80, age range 20-78 years] were recruited in the study. The patient group consisted of patients followed up and treated for IBD at the outpatient clinic between the dates March 2006 and January 2007. Diagnosis of UC was established using clinical, endoscopic and histological criteria. All patients underwent appropriate endoscopy (colonoscopy or rectosigmoidoscopy) to determine endoscopic activity indices and localization of disease. Biopsy samples were obtained for histopathologic examination when necessary. Rachmilewitz Endoscopic Activity Index was used to determine the degree of endoscopic activity[34]. Clinical activity index was determined according to Truelove and Witts criteria[35].

Current complaints, duration of disease, extraintestinal symptoms, smoking status, history of thromboembolic events (deep vein thrombosis, pulmonary emboli, myocardial infarction, stroke and peripheral arterial obstruction) and medications were recorded. Patients with any other systemic disorders including diabetes mellitus, hyperthyroidism, chronic liver and renal disease, and history of cancer, as well as patients on vitamin B12, folic acid and oral multivitamin supplements, were excluded from the study.

A detailed clinical history was obtained from all patients regarding any previous events of arterial or venous thrombosis. A total of 3 subjects (5.5%) had history of arterial and venous thromboembolic events. Two of these (3.63%) were peripheral deep vein thrombosis, and 1 (1.81%) was myocardial infarction (deep vein thrombosis was diagnosed using Doppler USG, and myocardial infarction using ECG and cardiac enzymes).

A healthy control group was composed of patients with similar age and sex distribution, and without any metabolic, neoplastic or inflammatory diseases or any history of thromboembolic diseases. The healthy control group consisted of a total of 45 healthy individuals [31 males, 14 females; mean age (± SD) 46.4 ± 13.89, age range 20-77 years].

Blood samples

Blood samples were obtained from all patients following 12-h fasting and these samples were used for complete blood count, sedimentation rate, C reactive protein (CRP), routine biochemical analysis (including total cholesterol, triglycerides, LDL cholesterol, VLDL cholesterol), thyroid function tests, folic acid, vitamin B12, homocysteine level examinations. Biochemical parameters were measured using standard methods. Serum levels of folic acid and vitamin B12 were measured using chemiluminescent microparticle immunoassay (CMIA) (Abbott System). Serum homocysteine levels were measured with high pressure liquid chromatography (HPLC) (Betamed, Agilent 1100 series, Chromosystems Reagent Kit).

Statistical analysis

Data were analyzed using SPSS 11.5 package software. Definitive statistics were indicated as mean ± SD or median (minimum-maximum) in continuous variables, whereas categorical data were expressed as number of observations (%). Presence of any significant differences between case and control groups in terms of measurements was evaluated using Mann Whitney *U* test. In contrast, Kruskal Wallis was used to determine any significant differences between treatment types, clinical activity index and localization groups in terms of Hcys levels. Spearman correlation test was used to determine the asso-

Table 1 Demographic features of UC and control groups

	Patients with UC (n = 55)	Healthy controls (n = 45)	P
Mean age (yr ± SD)	47.4 ± 13.80	46.4 ± 13.89	0.484
Gender (male/female)	38/17	31/14	0.983
Smoking status (%)	17 (30.9)	15 (33.3)	0.796
Localization (%)			
Rectum/sigmoid	34 (61.8)		
Left colitis	12 (21.8)		
Pancolitis	9 (26.4)		
Activity of UC (%)			
Active	28 (50.9)		
Inactive	27 (49.1)		
Medical treatment (%)			
5-ASA	41 (74.5)		
Steroids	3 (5.5)		
AZA	11 (20.0)		

UC: Ulcerative colitis; ASA: Aminosalicylic-acid; AZA: Azathioprine.

ciation of Hcys levels and duration of disease, clinical activity index, vitamin B12, folic acid and CRP. χ^2 or Fisher's exact test was used for categorical comparisons. A P level of < 0.05 was accepted for statistical significance.

RESULTS

Fifty-five patients with UC were recruited into the study. Patients consisted of 38 (69.1%) males, 17 (30.9%) females and mean age was 47.4 ± 13.80 years with a range between 20-78 years. Forty-five healthy individuals were recruited in the control group, consisting of 31 (68.9%) males, 14 (31.1%) females, with a mean age of 46.4 ± 13.89 years, range between 20-77 years. Groups did not differ statistically significantly in terms of age, sex and smoking status (P = 0.484, P = 0.983, P = 0.796, respectively).

Analysis of distribution of disease localizations in the UC group indicated that 34 (61.8%) patients had distal involvement, 12 (21.8%) patients had left colitis and 9 (16.4%) had pancolitis. Clinical activity indices of patients in the UC group showed that mild, moderate and severe cases were 28 (50.9%), 21 (38.2%) and 6 (10.9%) in number. Analysis of current treatment indicated that 41 patients (74.5%) received 5-ASA, 11 (20%) received azothioprine and 3 (5.5%) received corticosteroid treatment (Table 1).

Mean homocysteine level was 13.3 ± 1.93 μmol/L (range 4.60-87) in the UC group and 11.2 ± 3.58 μmol/L (range 4.00-20.8) in the control group ($P < 0.001$). Mean serum folic acid level was 5.1 ± 2.19 ng/mL (range 1.90-10.90) in the UC group and 6.3 ± 0.87 ng/mL (range 5-10) in the control group ($P < 0.001$). Mean level of vitamin B12 was 250.4 ± 82.49 pg/mL (range 96-505) in the UC group and 327.4 ± 73.90 pg/mL (range 200-482) in the control group ($P < 0.001$).

Thromboembolic events had similar prevalence in UC and control groups and did not differ statistically significantly in this respect (P = 0.250) (Table 2). It was determined that a total of 3 (5.5%) subjects had a history of arterial and venous thromboembolic events. Two

Table 2 Hcys, folic acid and vitamin B12 levels in UC and control groups (95% CI)

	Patients (n = 55)	Controls (n = 45)	P
Hcy (μmol/L)	13.3 ± 1.93	11.3 ± 3.58	< 0.001
Folic acid (ng/mL)	5.1 ± 2.19	6.3 ± 0.87	< 0.001
Vitamin B12 (pg/mL)	250.4 ± 82.49	327.4 ± 73.90	< 0.001
Thromboemboli	3 (5.5%)	0 (0%)	0.250

Table 3 Correlation between Hcys and other variables among patients with UC

	Relationship coefficient	P
Disease duration	0.105	0.447
Folic acid	-0.311	0.021
Vitamin B12	-0.146	0.288
CRP	0.244	0.073
Clinical activity index	0.115	0.403

of these (3.63%) were peripheral deep vein thrombosis and 1 (1.81%) was myocardial infarction (diagnoses were established using Doppler USG for deep vein thrombosis and ECG plus cardiac enzymes for myocardial infarction). The UC group did not differ from control to a statistically significant degree (P = 0.250). These three patients had normal Hcys levels.

No directly proportional relationship was determined in the UC group between homocysteine levels and disease duration, vitamin B12, CRP and clinical activity index; however, there was a negatively proportional relationship found between folic acid and homocysteine levels (r = -0.311 and P = 0.021) (Table 3).

There were no statistically significant differences within the disease localization groups of UC in terms of homocysteine levels (P = 0.096). Additionally, there were no statistically significant differences within the clinical activity index groups of UC in terms of homocysteine levels (P = 0.698). Similarly, there were no statistically significant differences within the treatment groups of UC in terms of homocysteine levels (P = 0.695).

DISCUSSION

Studies have shown that the risks of deep vein thrombosis and pulmonary emboli are three times higher in patients with IBD compared to the general population[4]. The exact pathogenesis of thromboembolism is not known and considered to be multifactorial[36-38]. Moderate hyperhomocysteinemia (hHcy) has been shown to be one of the independent risk factors associated with development of arterial and venous thrombosis[24,39-44].

Elevated Hcys might be due to genetic factors (MTHFR mutation), nutritional factors (deficiencies of folic acid, vitamin B6, vitamin B12) or medications (salazopyrin, methotrexate, corticosteroids)[28,45]. The most common cause of hHcy has been found to be folic acid deficiency in patients with UC[31,46,47].

Several previous studies also reported higher Hcys levels in patients with UC[28-33,45,48,49]. In our study, serum Hcys levels were higher in patients with UC than in healthy controls. Increased Hcys levels were negatively correlated with lower vitamin B12 and folic acid levels. However, multivariate regression analysis demonstrated that decreased folic acid levels were the most significant parameter in determining increased Hcys levels. In a similar study by Mahmut *et al*[28], increased Hcys levels were found to be associated particularly with folic acid deficiency. Hcys levels were observed to decrease following folate replacement and the authors recommended that folic acid levels should be determined and prophylactic folate treatment should be initiated in all patients with IBD. Zezoz *et al*[50] performed a similar study and found that the prevalence of increased Hcys was higher in the UC group compared to the control group (prevalence of Hcys was 30% in UC patients and 10% in control group). Statistical analysis showed that male sex, decreased folic acid and vitamin B12 levels were indicators of hHcy in patients with UC and that decreased folic acid levels were the most important factor. In our study also, decreased folic acid level was found to be the most important indicator of hHcy.

Folic acid deficiency might be associated with several factors in patients with IBD. These include inadequate intake, increased consumption or folate malabsorption in patients using medications, including particularly sulphasalazine (SASP)[30]. In the study of Zezos *et al*[50], one patient who was receiving SASP was found to have increased Hcys levels. An antifolate effect of SASP has also been demonstrated in other studies[28,29]. Only one patient was receiving SASP in our study and this patient had low folic acid and high Hcys levels.

Vitamin B12 levels were found to be decreased in our UC group compared to our control group; however this finding did not attain statistical significance. Studies have shown normal vitamin B12 levels among patients with increased Hcys levels[32,47]. The study of Romagnuolo *et al*[32] performed with regard to this issue has indicated that the prevalence of hHcy was 15.4% in IBD patients and that 80% of these patients had normal vitamin B12 levels. These authors suggested that there could be significant deficiency of vitamin depots or subclinical vitamin B12 deficiency in IBD patients with increased homocysteine and normal serum vitamin levels. In the study by Lambert *et al*[47], vitamin B12, B6 and folate deficiencies were determined as the most sensitive indicators of hHcy. These authors also suggested that subclinical vitamin deficiencies or intracellular vitamin deficiencies heralded vitamin deficiencies in circulation and that multivitamin (including folate) supplements were effective in decreasing Hcys levels. Similar to several previous studies, we found that hHcy did not correlate with disease activation, localization and medications[28,29,31,33]. However, some studies have shown a statistically significant relationship between disease activation and hHcys and reported that Hcys levels were markedly higher in patients with active disease compared to those with inactive disease[49,51].

Two recent studies have investigated Hcys levels in colon mucosa and aimed to discover the role of Hcys in the pathogenesis of UC[52,53]. Morgenstern *et al*[52] obtained biopsy samples from transverse colon and sigmoid or descending colon mucosa of 11 UC and 5 CD patients and examined these samples using high performance liquid chromatography (HPLC). Concentrations of Hcys were found to be higher in the UC and CD patients compared to healthy individuals. This study is the first to demonstrate elevated Hcys levels in human colon mucosa. No statistically significant differences were determined between Hcys levels, and disease activation or current treatment (particularly those receiving and not receiving sulphasalazine) in IBD patients. Interestingly, patients with other chronic inflammatory bowel diseases had normal Hcys levels; and patients with history of colorectal cancer had higher Hcys levels in colon mucosa compared to the normal[52]. The incidence of colon cancer among patients with UC is about 8 times higher than sporadic colon cancer. Hcys has mitogenic and proliferative features[54]. Elevated Hcys levels have been reported to be a risk factor for carcinogenesis[52].

In their study, Danese *et al*[53] measured both plasma and colon mucosal Hcys levels concomitantly in patients with IBD. They reported that Hcys levels were increased in both plasma and colonic mucosa of patients with UC and CD and suggested that Hcys could have a proinflammatory role in IBD. Similar to our study, this investigation did not determine any correlation between Hcys levels and disease activation or medications. A negative correlation was determined between circulatory and mucosal Hcys levels and folic acid levels, emphasizing the necessity of folic acid supplementation in patients with IBD[53].

Large retrospective studies have shown the rate of thromboembolic complications in patients with IBD to be between 1.3%-6.4%[55,56]. Oldenburg *et al*[29] reported that the rate of complications in a total of 231 patients with IBD was 5.7% in those with CD, and 9.0% in those with UC. Comparison of patients with and without venous thrombosis did not yield any statistically significant difference with regard to Hcys concentration. They suggested that hHcy did not influence the prevalence of thrombosis in patients with IBD; but that thrombosis developed secondary to multifactorial phenomena. These authors also suggested that hHcy could contribute to the pathogenesis of thromboembolic events in some of these patients[29].

Papa *et al*[33] reported the rate of thromboembolic complications to be 9.4% (6 patients) in 39 UC and 25 CD patients. There was hHcy in 2 of these patients. There was no statistically significant difference compared to the control group. Additionally, no statistically significant differences were found in Hcys levels in the within group comparison of IBD patients with regard to history of previous thromboembolic events[33]. In our study there were 3 patients with history of thromboembolic events and no statistically significant differences were present compared to the control group. We concluded that hHcy is not the major factor contributing to the development of thromboembolic complications; and that multifactorial etiology was more relevant.

In conclusion, hyperhomocysteinemia is a common

phenomenon in patients with IBD. Vitamin deficiencies should be determined in all patients with IBD and folate and vitamin B complex supplementations should be included in their treatment. In our study, we could not find any correlation between Hcys levels and history of arterial and venous thrombosis. Further studies should be performed to investigate the multifactorial etiology in the development of thromboembolic events in patients with IBD.

COMMENTS

Background

Patients with inflammatory bowel diseases (IBD) are at increased risk of experiencing thromboembolic complications. Studies have indicated a state of hypercoagulability involving all the components of the coagulation system in patients with ulcerative colitis (UC). The exact etiology and pathogenesis of hypercoagulability have not been explained, however, it is widely accepted that this condition is associated with acquired or genetic defects of the coagulation system and with the procoagulant effect of proinflammatory cytokines.

Research frontiers

In their study, Danese *et al* measured both plasma and colon mucosal homocysteine (Hcys) levels concomitantly in patients with IBD. They reported that Hcys levels were increased in both plasma and colonic mucosa of patients with UC and Crohn's disease (CD) and suggested that Hcys could have a proinflammatory role in IBD.

Innovations and breakthroughs

The results of studies investigating the influence of hyperhomocysteinemia on thrombosis in patients with IBD are controversial. Some studies suggest that it could contribute to the pathogenesis of thrombosis whereas other authors suggest that thrombosis develops in IBD patients secondary to multifactorial phenomena.

Applications

In this study, the authors found that the average Hcys level in UC patients was significantly higher than the average Hcys level of the control group. They could not find any correlation between Hcys levels and history of arterial and venous thrombosis. No directly proportional relationship was determined in the UC group between homocysteine levels and disease duration, vitamin B12, CRP level and clinical activity index; however there was a negatively proportional relationship found between folic acid and homocysteine levels. Vitamin B12 and folic acid average values were also lower in the UC group ($P < 0.001$). When multivariate regression analysis was performed, it was seen that folic acid deficiency was the only risk factor for hyperhomocysteinemia. For this reason the authors think that vitamin deficiencies should be determined in all patients with IBD and folate and vitamin B complex supplementation should be included in their treatment.

Terminology

Homocysteinemia is defined as elevation of homocysteine (a sulphur-containing amino acid) level in blood. It is an established risk factor for cardiovascular diseases and premature atherosclerosis.

Peer review

The study demonstrates increased levels of Hcys related to a decrease of folic acid and B12 in patients affected by UC. These data are not associated with an increase of thromboembolic events. The manuscript is well structured.

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BRIEF ARTICLE

eGFR is a reliable preoperative renal function parameter in patients with gastric cancer

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Abstract

AIM: To evaluate the validity of the estimated glomerular filtration rate (eGFR) as a preoperative renal function parameter in patients with gastric cancer.

METHODS: A retrospective study was conducted in 147 patients with gastric cancer. Preoperative creatinine clearance (Ccr), eGFR, and pre- and postoperative serum creatinine (sCr) data were examined. Preoperative Ccr and eGFR were then compared for their reliability in predicting postoperative renal dysfunction.

RESULTS: Among 110 patients with normal preoperative Ccr values, 7 (6.3%) had abnormal postoperative sCr values, and among 112 patients with normal preoperative eGFR values, postoperative sCr was abnormal in 5 (4.5%) (P = 0.53). Among 37 patients with abnormal preoperative Ccr values, 30 (81.1%) had normal postoperative sCr values, and of 35 patients with abnormal preoperative eGFR values, postoperative sCr was normal in 25 (71.4%) (P = 0.34). Preoperative Ccr was significantly correlated with eGFR (r = 0.514), and postoperative sCr was significantly correlated with preoperative Ccr (r = -0.334) and eGFR (r = -0.02).

CONCLUSION: Preoperative eGFR is as effective as Ccr for predicting postoperative renal dysfunction. eGFR should therefore be used as an indicator of preoperative renal function in place of Ccr since it is a cheaper and easier to perform test.

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Key words: Estimated glomerular filtration rate; Creatinine clearance test; Gastric cancer

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INTRODUCTION

The creatinine clearance (Ccr) test has been used as a gold standard for evaluation of preoperative renal function in patients with gastric cancer[1,2]. However, it is known that Ccr is not strictly equivalent to glomerular filtration rate (GFR) because of the intrinsic secretion of creatinine into urine from tubuli[3]. Furthermore, in order to measure Ccr, it is necessary to collect 2-h or

24-h urine samples, which is inconvenient in older patients and relatively expensive to implement.

Chronic kidney disease (CKD) is defined as a person having GFR less than 60 mL/min, and the frequency of CKD in the Japanese population is around 10%[4]. Therefore, in patients with gastric cancer, the detection of CKD before operation is a crucial matter to prevent postoperative renal complications. If there are other tests which reflect GFR more accurately and that can be performed easily and cheaply, this would be valuable in a clinical setting[5,6].

Although inulin clearance can be used for measuring GFR, it is not a standard preoperative renal function test because of its cumbersome methodology, and alternatively, the Ccr test has been used. In 1999, Levey *et al*[7] reported an equation for the estimation of GFR. The Modification of Diet in Renal Disease (MDRD) equation includes age, sex, race, serum creatinine (sCr) value, serum blood urea nitrogen value, and serum albumin value. It was suggested that the MDRD equation precisely reflects the GFR in Caucasians but not in Asians, and therefore an equation for estimating the GFR for Japanese individuals (eGFR) was postulated[8,9]. eGFR is calculated using three parameters: sex, age, and sCr. If eGFR is at least as precise as Ccr for estimation of postoperative renal dysfunction, it would be valuable as a preoperative renal function parameter for patients with gastric cancer, as eGFR is easier and cheaper to measure. To clarify this issue, therefore, we analyzed Ccr and eGFR retrospectively in a series of gastric cancer patients.

MATERIALS AND METHODS

One hundred and forty-seven patients diagnosed histologically as having gastric cancer who underwent surgery between January 2003 and December 2005, and for whom preoperative Ccr data were available, were enrolled in the present study. The patients' backgrounds are summarized in Table 1. Gastrectomy was performed based on the guidelines of the Japan Gastric Cancer Study Group[10]. Basically, patients took meals until lunch on the day before operation, and no hyperalimentation was performed. As antibiotics, 2 g/d of cephalosporin was administered from the day of the operation for 3 d.

Measurement of sCr was performed by standard enzymatic method. Ccr was measured by 24-h urine collection, and a value of less than 50 mL/min was regarded as abnormal. eGFR was calculated using the following equation[9]: eGFR (mL/min per 1.73 m^2) = $194Cr^{-1.094} \times Age^{-0.287}$ (× 0.739; for female patients). eGFR of less than 60 mL/min was regarded as abnormal. The upper normal limits of sCr for male and female patients were 1.1 mg/dL and 0.8 mg/dL, respectively.

The reliability of preoperative Ccr and eGFR for detecting postoperative renal dysfunction was analyzed.

Statistical analysis

Data were expressed as actual number of patients or mean ± SD. χ^2 test was used for two-group comparisons.

Table 1 Patients' backgrounds

Age (yr)	68.3 ± 9.8
Sex (M/F)	107/40
Pre-sCr (mg/dL)	0.74 ± 0.20
Pre-Ccr (mL/min)	71.5 ± 24.5
Pre-eGFR (mL/min)	71.1 ± 20.5
Post-max-sCr (mg/dL)	0.82 ± 0.30
Method of operation	
Distal gastrectomy	79
Total gastrectomy	59
Others	9

Pre-sCr: Preoperative serum creatinine; Pre-Ccr: Preoperative creatinine clearance; Pre-eGFR: Preoperative estimated glomerular filtration rate; Post-max-sCr: Maximum value of postoperative sCr.

Table 2 Preoperative Ccr and eGFR

	Normal	Abnormal	*P*-value
Pre-Ccr	37	110	0.89
Pre-eGFR	35	112	

There were no differences between Ccr and eGFR, with regard to abnormalities in preoperative tests.

Table 3 Distribution of postoperative sCr in patients with preoperative Ccr and eGFR abnormality

	Abnormal pre-Ccr	Abnormal pre-eGFR	*P*-value
Post-sCr			
Normal	8	5	0.29
Abnormal	9	12	

Postoperatively, 17 patients had abnormal sCr values. Of these 17 patients, 9 (52.9%) had abnormal preoperative Ccr values and 12 (70.6%) had abnormal preoperative eGFR values.

Statistical analyses were performed by using Graphpad software version 4.0. Significance of correlations was calculated by the Spearman correlation coefficient test. Differences at $P < 0.05$ were considered significant.

RESULTS

Preoperative Ccr was abnormal in 37 patients (25.2%) and preoperative eGFR was abnormal in 35 patients (23.8%) (Table 2, $P = 0.79$). Postoperatively, 17 patients had abnormal sCr values. Of these 17 patients, 9 (52.9%) had abnormal preoperative Ccr values and 12 (70.6%) had abnormal preoperative eGFR values (Table 3, $P = 0.29$).

Among the 110 patients who had normal preoperative Ccr values, 7 (6.3%) had postoperative sCr abnormality, and among the 112 patients who had normal preoperative eGFR values, 5 (4.5%) had postoperative sCr abnormality (Table 4, $P = 0.53$).

Among the 37 patients who had abnormal preoperative Ccr values, 30 (81.1%) had normal postoperative sCr values, and among the 35 patients who had abnormal preoperative eGFR values, 25 (71.4%) had normal postoperative sCr values (Table 5, $P = 0.34$).

Table 4 Distribution of postoperative sCr in patients with preoperative Ccr and eGFR normality

	Post-sCr		*P*-value
	Normal	Abnormal	
Normal pre-Ccr	103	7	0.53
Normal pre-eGFR	107	5	

Among the 110 patients who had normal preoperative Ccr values, 7 (6.3%) had postoperative sCr abnormality, and among the 112 patients who had normal preoperative eGFR values, 5 (4.5%) had postoperative sCr abnormality.

Table 5 Distribution of postoperative sCr in patients with preoperative Ccr and eGFR abnormality

	Post-sCr		*P*-value
	Abnormal	Normal	
Abnormal pre-Ccr	7	30	0.34
Abnormal pre-eGFR	10	25	

Among the 37 patients who had abnormal preoperative Ccr values, 30 (81.1%) had normal postoperative sCr values, and among the 35 patients who had abnormal preoperative eGFR values, 25 (71.4%) had normal postoperative sCr values.

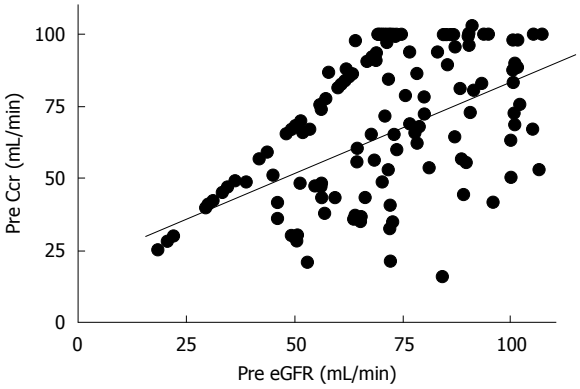


Figure 1 Correlation between preoperative creatinine clearance (Ccr) and estimated glomerular filtration rate (eGFR). Preoperative Ccr and eGFR were significantly correlated ($r = 0.514$, $P < 0.01$).

Next, we evaluated the correlation between preoperative Ccr, eGFR, and the maximum postoperative sCr value. Preoperative Ccr and eGFR showed a significant correlation ($r = 0.514$, $P < 0.01$, Figure 1). Preoperative Ccr was correlated with the maximum postoperative sCr value ($r = -0.334$, $P < 0.01$, Figure 2A). Also, preoperative eGFR was correlated with the maximum postoperative sCr value ($r = -0.188$, $P = 0.02$, Figure 2B).

There were no severe postoperative complications, such as anastomotic leakage, bleeding, or infection in the patients who had abnormal preoperative Ccr or eGFR values. None of the patients required postoperative hemodialysis.

DISCUSSION

The present study showed that eGFR was as equally reliable as Ccr for assessment of preoperative renal function in patients with gastric cancer.

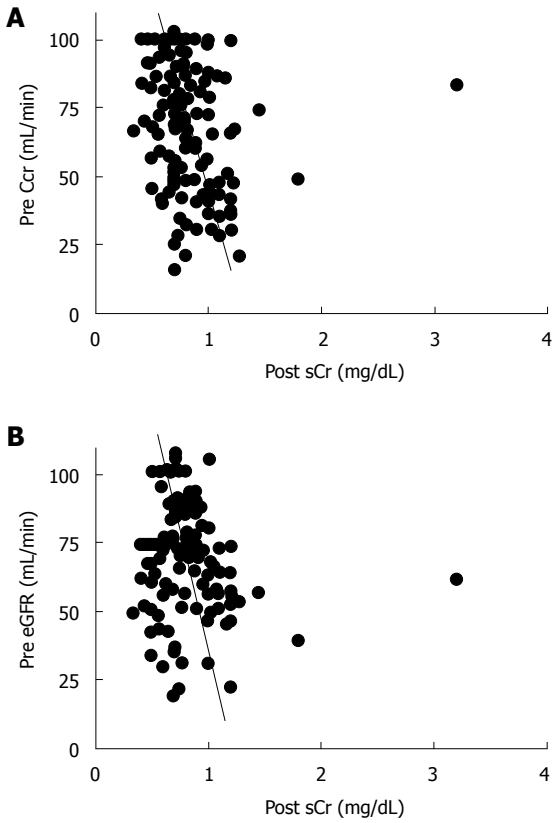


Figure 2 Correlation between preoperative Ccr and eGFR, and maximum postoperative serum creatinine (sCr). The maximum value of sCr was correlated with preoperative Ccr ($r = -0.334$, $P < 0.01$) (A) and eGFR ($r = -0.188$, $P = 0.02$) (B).

To date, Ccr has been used as a preoperative renal function parameter in patients with gastric cancer. Ccr is measured by 24-h or 2-h urine collection, and it has been suggested that either method can sometimes be inappropriate because of the difficulty involved in implementation, especially in older patients. Furthermore, there is a dissociation between Ccr and GFR in individuals with a low GFR[1]. Although sCr is a simply determined parameter for estimating renal function, it is not a reliable indicator of preoperative renal function because it is easily influenced by sex, muscle volume, and exercise. Also, sCr usually remains normal until GFR decreases to 50 mL/min[11].

GFR has not been used as a gold standard of preoperative renal function because measurement of inulin clearance is more labor-intensive than measurement of Ccr. However, the establishment of the MDRD equation by Levey *et al*[7] in 1999, and its modification postulated by the Japan Kidney Association, has made it possible to estimate GFR[8,9]. Therefore it is expected that eGFR will replace Ccr in various clinical settings.

In this study, we set the cut-off values of Ccr and eGFR at 50 mL/min and 60 mL/min, respectively. The validity of these cut-off values was endorsed by our finding that there was no significant difference in the number of patients who had abnormal postoperative sCr values between patients having preoperative Ccr ab-

normality and preoperative eGFR abnormality (Table 3).

There was no difference in the number of patients who had abnormal sCr values between patients who had normal preoperative Ccr values and normal preoperative eGFR values (Table 4), and also no difference in the number of patients who had normal sCr values between patients with preoperative Ccr abnormality and preoperative eGFR abnormality (Table 5). These results indicate that there is no significant difference in reliability between preoperative Ccr and eGFR for detecting postoperative renal dysfunction.

Preoperative Ccr and eGFR values showed a significant correlation (Figure 1), and Ccr and eGFR were correlated with the maximum postoperative sCr value (Figure 2). Thus, either Ccr or eGFR can be used to predict the maximum postoperative sCr value.

Cystatin C is a cysteine protease and a member of the cystatin super family. Cystatin C is excreted from the glomerulus and reabsorbed by the proximal tubuli, and has been used as a marker for GFR[12,13]. sCr is influenced by prerenal factors, such as muscle volume; on the other hand, cystatin C is not influenced by sex, age, and other prerenal factors. In this retrospective study, cystatin C was not included as a parameter; it would be valuable to evaluate the relationship between eGFR and cystatin C as a preoperative renal function test of gastric cancer.

We conclude that eGFR is as equally valuable as Ccr as an indicator of preoperative renal function in patients with gastric cancer, and that eGFR may now be used in place of Ccr in view of the former's clear medical and socioeconomic advantages.

COMMENTS

Background

Creatinine clearance (Ccr) is not strictly equal to glomerular filtration rate (GFR). It has been accepted that estimated GFR (eGFR) is equal to measured GFR in chronic kidney disease. However, there have been no previous studies regarding the reliability of eGFR as a preoperative renal function test in gastric cancer patients.

Research frontiers

If eGFR is as good as Ccr as a preoperative renal function test, eGFR may replace Ccr because of its simplicity of measurement.

Innovations and breakthroughs

eGFR is useful as a preoperative renal function parameter in patients undergoing gastrectomy. Ccr is no longer recommended as a first-choice preoperative renal function test.

Applications

eGFR should replace Ccr as a routine preoperative renal function test in various surgical fields.

Terminology

eGFR is estimated GFR which is calculated from age, sex, serum creatinine value (eGFR3), or by adding serum albumin concentration and blood urea nitrogen value (eGFR5).

Peer review

I think the conslusion should state "may be as good" not be as categorical as eGFR should be used instead of Ccr. This study based on a 147 patient is a good proof of principle but certainly does not have the power to change the standard of care.

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BRIEF ARTICLE

EZH2 and STAT6 expression profiles are correlated with colorectal cancer stage and prognosis

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Abstract

AIM: To investigate the role of enhancer of zeste homologue 2 (EZH2) and STAT6 immunohistochemistry in the evaluation of clinical stages and prognosis of colorectal cancer (CRC).

METHODS: The expression patterns were examined by immunohistochemistry in both tumor and adjacent non-neoplastic tissues of 119 CRC patients who underwent operation during the time period from 2002 to 2004.

RESULTS: The positive rates of EZH2 and STAT6 in CRC cases were 69.7% (83 of 119) and 60.5% (72 of 119), respectively, and there was significant difference when compared with tumor adjacent non-neoplastic tissues ($P < 0.05$). In all CRC cases, patients with EZH2-positive, or STAT6-positive expression had lower survival rates than those with EZH2-negative or STAT6-negative expression ($P = 0.002$ and $P = 0.005$, respectively). Co-expression of EZH2 and STAT6 showed significantly higher levels in CRC cases of high clinical TNM stages ($P = 0.001$), and the expression of STAT6 was also correlated with lymph node metastasis and distant metastasis ($P = 0.001$ and $P = 0.016$, respectively). Multivariate analysis revealed that EZH2 expression was an independent prognostic indicator of CRC ($P = 0.039$).

CONCLUSION: EZH2 and STAT6 expressions have significant values in distinguishing clinical stages of CRC and predicting the prognosis of the patients.

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Key words: Colorectal Neoplasms; Enhancer of zeste homologue 2; STAT6; Immunohistochemistry

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INTRODUCTION

Colorectal cancer is the third most common cancer, and the second leading cause of death from cancers in the United States[1]. Many Asian countries, including China, Japan, South Korea, and Singapore, have experienced an increase of 2-4 times in the incidence of CRC during the

past few decades[2]. To reduce the mortality and improve treatment, a number of studies have been performed to search for tumor biomarkers, especially for those highly correlated with both staging and prognosis that can help not only predict patients' survival, but also lead to optimal therapeutic project.

EZH2 is a member of the polycomb group of genes (PcG), which is important for transcriptional regulation through nucleosome modification, chromatin remodeling, and interaction with other transcription factors[3]. EZH2, also called histone lysine methyltransferase (HKMT), has the function of methylating lysine 9 and 27 of histone H3, which consequently leads to the repression of target gene expression. Furthermore, dysregulation of this gene silencing mechanism may lead to cancer[4,5]. Recently, EZH2 has been investigated as a potential molecular biomarker for the diagnosis and prognosis of prostate cancer by Varambally *et al*[6], using cDNA microarray analysis. Overexpression of EZH2 has been observed in the most aggressive forms of prostate cancer, exhibiting a correlation with poor clinical outcome, thus acting as a novel marker of aggressiveness and unfavorable prognosis in this type of cancer. A causal effect between EZH2 up-regulation and poor prognosis was also observed in human gastric cancers[7]. However, there have been few reports of EZH2 expression in CRC, and its relationship with clinicopathological characteristics or prognostic value is yet unclear.

Like other members of the signal STAT (transcriptional activation by signal transducer and activator of transcription) family of proteins, STAT6 has a dual role as a signaling molecule and transcription factor. STAT6 is activated in response to interleukin (IL)-4 and IL-13 stimulation, and plays a key role in Th2 polarization of the immune system[8]. Recently, the STAT6 signaling pathway was found to be highly activated in some tumors, such as prostate cancer, mammary carcinoma and lymphoma[9-11]. In addition, a close correlation between IL-4/STAT6 activities and apoptosis and metastasis of colon cancer was reported by Li *et al*[12] in a study using STAT6-high and STAT 6-null colon cancer cell lines to analyze anti-apoptotic and pro-metastatic genes expression. Nevertheless, there have been no studies investigating the clinicopathological features and prognosis of CRC in relation to STAT6 expression. In this study, we analyzed the expression of EZH2 and STAT6 in biopsied CRC tissues to determine their relationship with clinicopathological features and clinical outcome of CRC patients.

MATERIALS AND METHODS

Patient and follow-up

A group of 119 consecutive patients with CRC were studied. All patients were diagnosed and treated in the Beijing People's Hospital between December 2002 and June 2004. There were 74 men and 45 women with a mean age of 62.2 ± 12.8 years (range 25-89 years). No patient had received preoperative chemotherapy or radiotherapy. All of the patients were followed up by direct evaluation or phone interview until death or December 2008. This study was approved by the Ethics Committee of People's Hospital, Peking University.

Tissue samples and tissue array

Core tissue biopsies (2 mm in diameter) were taken from Formalin fixed paraffin-embedded tumor samples and adjacent non-neoplastic tissues. These blocks were arranged in a new recipient paraffin block (tissue array block) using a commercially available micro-array instrument (Beecher Instruments, Micro-Array Technologies, Silver Spring, MD). Two cores were sampled from each case. From the 119 tumor samples and adjacent non-neoplastic tissues, 11 tissue array blocks were prepared, each containing 10-12 tumor and adjacent non-neoplastic sample cores. To qualify for this study, a case needed to have a tumor occupying more than 10% of the core tissue area. Clinical and pathological information for this group of patients was obtained by review of histopathological reports and medical records.

Immunohistochemistry

Eleven paraffin-embedded blocks of tissue arrays were cut into 4-mm sections. The sections were put in the oven at 59℃ for 1h, deparaffinized in xylene, rehydrated in a graded ethanol series, and treated with 3% hydrogen peroxide solution for 10 min. Antigen retrieval was done by microwaving the tissue in 10 mmol/L citric acid buffer for 10 min, then cooling to room temperature for 1 h. The sections were incubated with an anti-EZH2 monoclonal antibody (1:500, Invitrogen), and an anti-STAT6 monoclonal antibody (1:100, Abcam) overnight at 4℃. Primary antibodies were detected using the Powervision two-step histostaining reagent (Zhongshan, Beijing), with PV-6001 as the secondary antibody and detection was performed by diaminobenzidine (DAB) chromogenic reaction. Tissues were counterstained with hematoxylin, 1% hydrochloric acid of alcohol, dehydrated with graded ethanols, and mounted. Positive and negative immunohistochemical controls were included. Two experienced pathologists independently examined EZH2 and STAT6 staining while blinded to the clinicopathological data and clinical outcomes of the patients. Cases with > 30% positive tumor cells in a section were recorded as having positive expression. As negative controls, samples were stained with only secondary antibody. A total of 119 cases were used for data analysis.

Statistical analysis

All data were analyzed using SPSS 11.0 software. The association of EZH2 and STAT6 expression with various clinicopathological features was analyzed using χ^2 test. Cumulative survival was estimated by the Kaplan-Meier method and differences between survival curves were analyzed by log-rank test. The influence of each variable

Table 1 Relationship between EZH2 or STAT6 and clinicopathological features

	Cases	EZH2 expression		*P*	STAT6 expression		*P*
		EZH2+	EZH2-		STAT6+	STAT6-	
Tissue							
Non-neoplastic samples		22	97	0.000	50	69	0.004
Tumor samples		83	36		72	47	
Gender							
Female	47	30	17	0.334	24	23	0.089
Male	72	52	20		48	24	
Location							
Colon	90	62	28	0.719	57	33	0.268
Rectum	29	21	8		15	14	
Differentiation							
Poor	21	16	5	0.479	16	5	0.101
Moderate or well	98	67	31		56	42	
TNM classification							
Ⅰ	11	4	7	0.049	3	8	0.004
Ⅱ	42	29	13		20	22	
Ⅲ	44	35	9		32	12	
Ⅳ	22	15	7		17	5	
Depth of wall invasion							
T1	1	1	0	0.014	1	0	0.188
T2	19	9	10		8	11	
T3	93	71	22		58	35	
T4	6	2	4		5	1	
Lymph node metastasis							
No	55	34	21	0.081	25	30	0.001
Yes	64	49	15		47	16	
Distant metastasis							
M0	97	66	31	0.668	54	43	0.024
M1	22	16	6		18	4	
Recurrence							
No	86	61	25	0.088	53	33	0.775
Yes	11	6	5		4	7	

on survival was analyzed by multivariate analysis using the Cox proportional hazard model. Differences with $P < 0.05$ were considered statistically significant.

RESULTS

EZH2 and STAT6 expression

Positive and negative staining of EZH2 and STAT6 are illustrated in Table 1. The positive rates of EZH2 and STAT6 were 69.7% (83 of 119) and 60.5% (72 of 119), respectively in CRC cases, and there was an obvious difference when compared with tumor adjacent non-neoplastic tissues ($P < 0.05$). We observed that EZH2 expression was mainly present in the nuclei in most of the cases, and EZH2 immunoreactivity was found not only in the nuclei but also in the cytoplasm in a few CRC samples (8 of 110). STAT6 expression was mostly detected in the cytoplasm, and at cell membranes in some cases (Figure 1).

The expression of EZH2 in CRC samples was significantly correlated with more malignant phenotypes, including TNM classification ($P = 0.049$), and tumor invasion depth ($P = 0.014$). The expression of EZH2 had nothing to do with gender, tumor location, differentiation, lymph node metastasis, or distant metastasis. There was no significant correlation between cytoplasmic EZH2 immunoreactivity and clinicopathological features in the CRC carcinoma tissue samples (data not shown).

We found a positive correlation between STAT6 expression and TNM classification ($P = 0.004$), and expression of STAT6 was significantly associated with lymph node metastasis ($P = 0.001$) and distant metastasis ($P = 0.024$). As shown in Table 1, expression of STAT6 was not correlated with other clinicopathological features.

Prognostic implications of EZH2 and STAT6 expression in CRC

Tissue biopsies with EZH2-positive cells had a significantly lower post-operative survival rate than tissue biopsies with EZH2-negative cells ($P = 0.02$, Figure 2A). The STAT6-positive group also had significantly lower survival rates than the STAT6-negative group ($P = 0.005$, Figure 2B).

On the basis of the expression profiles of EZH2 and STAT6, the 119 patients were categorized into three groups: Group A: EZH2-/STAT6- ($n = 30$); Group B: EZH2+/STAT6- or EZH2-/STAT6+ ($n = 40$); and Group C: EZH2+/STAT6+ ($n = 59$). There were significant differences in survival rates between group A and one of the other groups ($P = 0.006$, Figure 2C). The survival rate of group A was significantly higher than that of group B ($P = 0.008$), the survival rate of group B was not

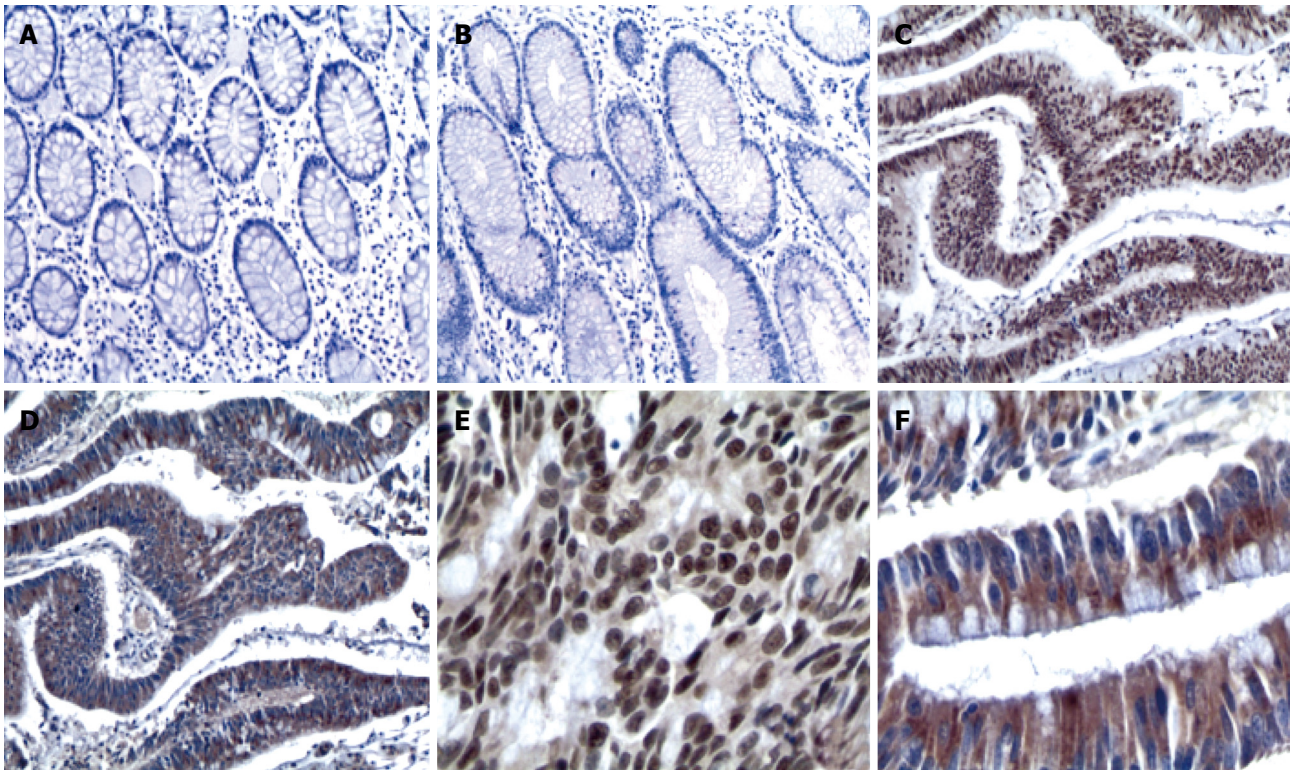


Figure 1 Expression of EZH2 (A, C, E) and nuclear STAT6 (B, D, F) in adjacent non-neoplastic tissues and CRC tissues visualized by DAB. A: Non-neoplastic tissues stained with EZH2; B: Non-neoplastic tissues stained with STAT6; C, E: Tumor tissues stained with EZH2; D, F: Tumor tissues from the same case stained with STAT6. Original magnifications: × 100 (A-D); × 400 (E, F).

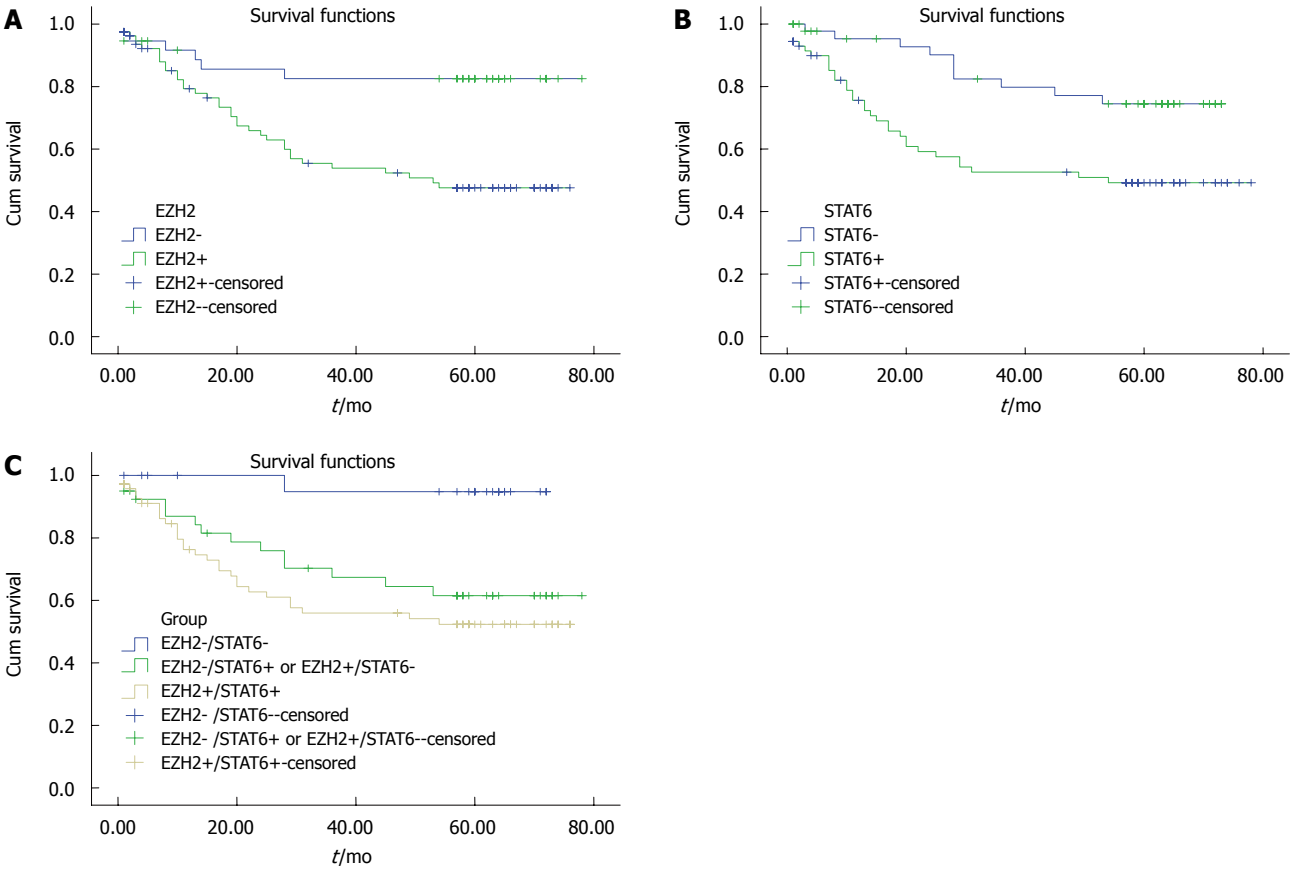


Figure 2 Kaplan-Meier survival curves for 119 CRC patients categorized by EZH2 and/or STAT6 expression. A: Survival was significantly lower in patients with positive EZH2 expression than in those with negative expression (P = 0.02); B: Survival was significantly lower in patients with positive STAT6 expression than in those with negative expression (P = 0.005); C: Patients with EZH2+/STAT6+ expression profiles had worse survival outcome (P = 0.006).

Table 2 Multivariate analysis of prognostic factors in 119 cases using the Cox proportional hazard model

	B	*P*	RR	95% CI for RR	
				Lower	Upper
Metastasis	3.075	0.000	21.648	8.212	57.070
Lymph node metastasis	1.149	0.018	3.157	1.218	8.178
Grading	-0.675	0.051	0.509	0.258	1.003
Recurrence	0.510	0.173	1.665	0.799	3.469
EZH2	1.167	0.039	3.214	1.061	9.732

B: Regression coefficient.

Table 3 Relationship between EZH2 and STAT6 expressions

EZH2	STAT6		Total
	+	-	
+	59	24	83
-	13	23	36
Total	72	47	119

r_s = 0.329, *P* = 0.00.

significantly higher than that of group C (*P* = 0.333), and the survival rate of group A was significantly higher than that of group C (*P* = 0.001, Figure 2C).

Using Cox regression analysis for the 119 patient samples, positive EZH2 expression (*P* = 0.039), distant metastasis (*P* = 0.000), and lymph node metastasis (*P* = 0.018) seemed to be independent prognostic indicators (Table 2).

Combined analysis of EZH2 and STAT6 expression in distinguishing TNM classification

A significant association was found between EZH2 and STAT6 expression by Spearman's correlation analysis (r_s = 0.329, *P* = 0.00; Table 3), when EZH2 and STAT6 expressions were evaluated. Table 4 shows the relationship between the expression profile of EZH2 and STAT6 and TNM classification assignment for 119 CRC samples. Co-expression of EZH2 and STAT6 was significantly correlated with high clinical TNM classification (*P* = 0.003). Furthermore, the expression levels of co-expression of EZH2 and STAT6 tended to rise as the TMN staging increased (18%, 40%, 59% and 72% in stages I -Ⅳ, respectively).

DISCUSSION

In the present study, we have found that EZH2 and STAT6 are sensitive and potential biomarkers that can be used for the prediction of clinical TNM classification and prognosis of CRC. An association between EZH2 overexpression and the biological malignancy of tumors has been reported in many cancers, including breast cancer, lymphoma, bladder carcinoma and hepatocellular carcinoma. Those studies suggested that EZH2 may play a role in carcinoma progression and indicate

Table 4 Relationship between EZH2 and STAT6 co-expression and TNM classification

TNM classification	EZH2 and STAT6 expressions				EZH2+ and STAT6+ expression in different stages (%)
	EZH2+ and STAT6+	EZH2- or STAT6-	EZH2- and STAT6-	Total	
Ⅰ	2	3	6	11	18
Ⅱ	15	19	8	42	40
Ⅲ	26	10	8	44	59
Ⅳ	16	5	1	22	72
Total	59	37	23	119	49

P = 0.003.

a worse clinical outcome[13-16]. Fluge *et al*[17] investigated the expression of EZH2 in colorectal cancer by IHC. The authors concluded that a strong EZH2 expression indicated an improved relapse-free survival. Notably, this finding was only seen in colonic cancer, but not in rectal cancer. Interestingly, this result is conflicted with Mimori' study[18], who believes that EZH2 may be an oncogene and a prognostic marker in either colonic or rectal cancer. In our study, no relationship was found between the expression of EZH2 and colorectal tumor location. Furthermore, those CRC cases with higher levels of EZH2 expression were of more malignant phenotypes and have poorer prognosis.

The underlying mechanism is related, most probably, to tumor suppressor gene silencing by EZH2 mediated histone methylation and DNA methylation[19]. We found a positive correlation between EZH2 expression and TNM staging. Multivariate analysis revealed that EZH2 represents an independent prognostic indicator. These findings suggest the potential clinical utility of incorporating EZH2 into clinical consideration to help determine the clinical TNM staging and predict the outcome.

Our data showed that STAT6 expression was positively related to clinical TNM staging, lymph node metastasis and distant metastasis, suggesting that STAT6 may promote the progression of cancer. STAT6 is a member of STAT family of latent transcription factors known to be activated constitutively in cancers[20]. STAT6 is activated in response to interleukin (IL)-4 and IL-13 stimulation, STAT6-/-mice were deficient in responsiveness to IL-4 and IL-13, hence preferentially producing CD4_Th1 cells rather than CD4_Th2[21]. This observation may indicate that STAT6-deficient mice might have heightened immuno-surveillance against primary and metastatic tumors, because the development of Th1 cells optimized CD8-mediated tumor immunity[22]. Our data support these mechanism studies about STAT6, and suggest that STAT6 plays a key role in progression and metastasis of CRC.

In this study, STAT6 expression was also found to be associated with a poorer prognosis and TNM classification, which was consistent with EZH2 expression. Consequently, STAT6 enhanced the predictive efficiency of EZH2 expression, and patients with positive expres-

sion of EZH2 and STAT6 had the worse outcome and higher TNM staging than patients with negative expression of EZH2 or STAT6. These results suggest that combined analysis of EZH2 and STAT6 may be a powerful biological marker to predict clinical TNM staging and prognosis. According to the NCCN guidelines, patients with different clinical stages should receive correspondingly optimal treatment protocols. Therefore, it is critical to find effective biomarkers which can predict clinical staging in order to enable clinicians to make a more precise prejudgment. Our data suggest that combined EZH2 and STAT6 analysis may have a potential ability for this purpose.

In conclusion, the prognosis of CRC patients with EZH2-positive or STAT6-positive expression is significantly worse than those CRC patients with EZH2-negative or STAT6-negtive expressions. In addition, combined analysis of EZH2 and STAT6 expressions could enable clinicians to prejudge a more accurate clinical TNM staging before operation.

COMMENTS

Background

Enhancer of zeste homologue 2 (EZH2) is a member of the polycomb group of genes that is involved in epigenetic silencing and cell cycle regulation. Recently, EZH2, as well as STAT6 signaling pathway, were found to be highly activated in some tumors. However, the clinical significance of these proteins has not yet been determined in colorectal cancer (CRC).

Research frontiers

Recently, EZH2 has been under investigation as a potential molecular biomarker for the diagnosis and prognosis in prostate cancer. Overexpression of EZH2 has been observed in the most aggressive forms of prostate cancer, exhibiting a correlation with poor clinical outcome, thus acting as a novel marker of aggressiveness and unfavorable prognosis in this type of cancer. A causal effect between EZH2 up-regulation and poor prognosis was also observed in human gastric cancer. STAT6 has a dual role as a signaling molecule and transcription factor. STAT6 signaling pathway was found to be highly activated in some tumors, such as prostate cancer, mammary carcinoma and lymphoma. In addition, a close correlation between IL-4/STAT6 activities and apoptosis and metastasis of colon cancer was reported.

Innovations and breakthroughs

The authors found that patients with EZH2-positive expression had lower survival rates than those with EZH2-negative expression in both colonic and rectal cancers. Furthermore, the data suggest that combined analysis of EZH2 and STAT6 expression can be of significant value in distinguishing clinical stages of CRC and predicting prognosis.

Applications

The combined analysis of EZH2 and STAT6 may be a powerful biological marker, which could enable clinicians to prejudge a more accurate clinical TNM staging and prognosis of CRC patients before operation.

Terminology

EZH2 is a member of the polycomb group of genes (PcG), which is important for transcriptional regulation through nucleosome modification, chromatin remodeling, and interaction with other transcription factors. EZH2, also called histone lysine methyltransferase (HKMT), has the function of methylating lysine 9 and 27 of histone H3, which consequently leads to the repression of target gene expression.

Peer review

Generally, the paper is well written and designed.

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BRIEF ARTICLE

Expression of calreticulin is associated with infiltration of T-cells in stage ⅢB colon cancer

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Abstract

AIM: To investigate the correlation between expression of calreticulin and infiltration of lymphocytes in stage ⅢB colon cancer.

METHODS: Sixty-eight pathologically-confirmed specimens were obtained from stage ⅢB (T3N1M0) colon cancer patients who underwent radical resection between January 1999 and May 2002 at the Cancer Center of Sun Yat-Sen University, Guangzhou, China. Immunohistochemical analysis was performed to show infiltration of lymphocytes and expression of calreticulin in colon cancer. Association between calreticulin expression, infiltration of lymphocytes, and 5-year survival rate of patients was assessed.

RESULTS: The expression level of calreticulin was lower in cancer nest than in its adjacent normal epithelium since 61.8% (42/68) of the samples were stained with calreticulin in colon cancer. The expression of calreticulin in colon cancer was associated with the infiltration of CD45RO+ cells rather than with that of CD3+ cells. In addition, the stronger expression of calreticulin and the higher infiltration of CD3+ and CD45RO+ cells in colon cancer were associated with the higher 5-year survival rate of patients.

CONCLUSION: Expression of calreticulin is associated with infiltration of T-cells, which implies that a low expression level of molecular marker may represent a new mechanism underlying immune escape in colon cancer.

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Key words: Calreticulin; Tumor-infiltrating lymphocyte; Colon cancer; Immune escape

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INTRODUCTION

Mounting evidence indicates that colorectal cancer is immunogenic[1]. For example, when immunologic effector cells, such as CD3+ T-cells, CD45RO+ T-cells and macrophages, infiltrate colorectal cancer tissue, tumor progression is decreased[2-7]. However, colorectal cancer cells have developed multiple immune escape mechanisms, such as reduced expression of HLA-I molecules, expression of immune inhibitors including TGF-β and indoleamine 2,3-dioxygenase (IDO), and induction of Treg cells[8-11]. More importantly, targeting the immune system can stimulate an immune response in colorectal cancer[12-16].

The mechanisms by which colorectal cancer cells induce an immunologic response remain unknown. Recently, evidence indicates that a subset of damage-associated molecular patterns (DAMPs) is involved in the induction of immune response. During the apoptosis of cancer cells, translocation of calreticulin (CRT) from endoplasmic reticulum (ER) to membrane (ecto-CRT exposure), expression of heat-shock proteins, release of HMGB1 from cells, and expression of NKG2D may serve as danger signals, thereby inducing an immune response[17,18]. CRT is a highly conserved 46 kDa Ca^{2+}-binding protein, which is mainly located in the lumen of ER and has various versatile functions, such as chaperone activity, regulation of Ca^{2+} homeostasis, adhesion signaling, involving gastrointestinal mucin synthesis at the stage of folding and oligomerization in ER, inhibiting angiogenesis and tumor growth[19-23]. Within ER, CRT interacts with various molecules like ERp57 and calnexin (CNX) to aid in proper folding of proteins[24]. Furthermore, CRT plays an important role both in the assembly of MHC class I molecule and in the loading of antigen peptides onto the MHC- I molecule within ER[25]. Besides, it has been shown that nuclear CRT can regulate nuclear protein transport and influence signaling *via* nuclear steroid receptors and integrins[26,27]. When cells are treated with anthracyclines, oxaliplatin, radiation and hypoxia, ecto-CRT exposure works as an "eat me" signal for dendritic cells and macrophages, initiating an immune response[28-30].

Altered expression of calreticulin has been detected in melanoma, and in liver, bladder, prostate, lung, pancreatic and breast cancers[31-37]. However, the clinical significance of CRT expression remains poorly understood and controversial. It has been reported that overexpression of CRT is related to the promotion of breast cancer and decreases the progression of prostate cancer[34,36]. Additionally, interaction between calnexin and calreticulin contributes to metastasis of melanoma[31]. Since the clinical evidence supporting the immune regulation of CRT is scant, this study examined the expression of CRT in stage ⅢB colon cancer patients to evaluate whether the expression of CRT is associated with the immunogenicity of colon cancer.

MATERIALS AND METHODS

Materials

Sixty-eight pathologically-confirmed specimens were obtained from patients with stage ⅢB (T3N1M0; AJCC, 2002) colon cancer between January 1999 and May 2002 at the Cancer Center of Sun Yat-Sen University, Guangzhou, China (Table 1). All the patients underwent radical resection and 5-FU-based adjuvant chemotherapy after operation for 6 mo. The patients were evaluated every 3 mo during the first year, every 6 mo in the second year, and once every year thereafter for a total of 5 years. If a recurrence or a metastasis occurred, 5-FU-based chemotherapy was given according to the national comprehensive cancer network (NCCN) guidelines. No patients received preoperative blood transfusion or non-steroidal anti-inflammatory drugs. Overall survival was defined as the time from surgery to death. Data analysis was done on the last known day when the patient was alive.

Immunohistochemical assay and scoring systems

Formalin-fixed, paraffin-embedded tissue was cut into 4-μm thick sections. The size of each tissue section was about 1 cm × 1 cm. Then, the sections were dewaxed, rehydrated, and blocked with hydrogen peroxide. Antigens were retrieved in 10 mmol/L citrate buffer (pH 6.0) for 10 min and cooled to room temperature. After blocked with sheep serum, the sections were incubated overnight at 4℃ with either rabbit polyclonal antibody against human calreticulin at a dilution of 1:2000 (Abcam, Cambridge, MA, USA) or mouse monoclonal antibody against human CD3 and CD45RO (Zymed, San Diego, CA, USA), both of which were diluted to 1:100. Subsequently, biotinylated secondary antibodies and streptavidin-biotinylated horseradish peroxidase complexes were used. The sections were developed with diaminobenzidine tetrahydrochloride (DAB) and counterstained with hematoxylin. Negative controls in which primary antibody was replaced with a phosphate buffered solution (PBS), were employed.

Infiltration of lymphocytes in the tumor was scored with Hussein's method[38] and expression of calreticulin in colon cancer was interpreted *via* immunoreactivity using the 0-4 semi-quantitative system derived from Remmele and Stegner for both the intensity of staining and the percentage of positive cells (labeling frequency percentage)[39]. The cells were counted in at least 10 different fields for each section, and the size of each high-power field (× 400) was about 300 μm × 300 μm. The cells were counted in tumor stroma. The highest infiltration areas of lymphocytes were chosen. Necrotic areas were avoided. Two observers counted the cells at the same time and in the same field under a multiple-lens microscope. The results were expressed as mean ± SE. Cytoplasm staining

Table 1 Parameters of patients (n = 68)

Parameters	n (%)
Age (yr)	
< 60	30 (44.1)
⩾ 60	38 (55.9)
Gender	
Male	38 (55.9)
Female	30 (44.1)
Tumor site	
Left hemicolon	45 (66.2)
Right hemicolon	23 (33.8)
Pathological grade	
G1	10 (14.7)
G2	51 (75.0)
G3	7 (10.3)
Survival time (mo)	
⩾ 60	52 (76.5)
< 60	16 (23.5)

was divided into no staining/background of negative controls (score = 0), weak staining above background (score = 1), moderate staining (score = 2), and intense staining (score = 3). The labeling frequency was scored as 0 (⩽ 1%), 1 (1%-24%), 2 (25%-49%), 3 (50%-74%), and 4 (⩾ 75%), respectively. The product index was obtained by multiplying the intensity and percentage and scored as (-), (+), (++), and (+++), which indicate the cross-indices of 0-2, 3-5, 6-8, and 9-12, respectively. (-) was defined as no or negative expression, and (+)-(+++) as positive expression. Each section was independently scored by two pathologists. If an inconsistency occurred, a third pathologist was consulted to achieve a consensus.

Statistical analysis

Correlation between calreticulin expression, or infiltration of lymphocytes and parameters of patients was analyzed by χ^2 test or Fisher's exact test. Factors, including gender and age of the patients, pathologic grade, tumor site, infiltration of CD3+ cells and CD45RO+ cells, and calreticulin expression level in colon cancer, were assessed by univariate and multivariate analysis to determine their influence on the overall survival rate of patients. Kaplan-Meier curve and log-rank test were used to estimate the distribution of variables in relation to survival. Cox regression model was used to correlate the assigned variables with the overall survival rate. All statistical analyses were carried out using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

RESULTS

Expression of CRT and infiltration of lymphocytes in stage ⅢB colon cancer

CRT was stained in cytoplasm rather than in nuclei of cancer cells and normal epithelium. The expression level of CRT was lower in colon cancer than in its adjacent normal epithelium. Of the stained samples, 61.8% (42/68) were positive for CRT in cancer nest (Figure 1A and B,

Table 2 Relation between expression of calreticulin, infiltration of lymphocytes and parameters of patients (n = 68)

Parameters	Calreticulin expression		P
	(-)	(+)-(+++)	
Gender			0.813
Male	15	23	
Female	11	19	
Age (yr)			0.204
⩾ 60	12	26	
< 60	14	16	
Tumor site			0.245
Left hemicolon	15	30	
Right hemicolon	11	12	
Pathological grade			0.643
G1	5	5	
G2	19	32	
G3	2	5	
Infiltration of CD3+ cells per high-power field			0.387
> 18	18	33	
⩽ 18	8	9	
Infiltration of CD45RO+ cells per high-power field			0.010
> 26	15	36	
⩽ 26	11	6	

Table 2). CD3+ and CD45RO+ cells were observed in all samples from tumor stroma and its adjacent normal mucosa. Positively stained antigens were found on cell membrane (Figure 1C-F).

Relation between expression of CRT and infiltration of CD3+ or CD45RO+ cells

The cut-off value for infiltration of lymphocytes in colon cancer was 75%. Infiltration of 18 CD3+ cells and 26 CD45RO+ cells was observed in per high-power field, and was thus recorded as high and low infiltration. Log-rank test was used to analyze the relation between expression of CRT and infiltration of CD3+ and CD45RO+ cells in colon cancer, showing that positive expression of CRT (+-+++) was associated with high infiltration of CD45RO+ cells (P = 0.010, Table 2). No correlation was observed between expression of CRT and infiltration of CD3+cells or other parameters of the patients, such as age, gender, tumor site.

Univariate and multivariate survival analysis

By the end of a 5-year follow-up period, 52 patients were alive with a 5-year survival rate of 76.5%. Kaplan-Meier survival analysis indicated that positive expression of CRT was associated with a higher 5-year survival rate. The 5-year survival rate of patients with positive and negative expression of CRT was 85.5% (36/42) and 61.5% (16/26), respectively (P = 0.022, Table 3). However, the survival curves for patients with positive and negative expression of CRT were crossed at 18 mo (Figure 2). In addition, high infiltration of CD3+ and CD45RO+ cells in colon cancer was associated with a higher 5-year survival rate (P = 0.000, Figure 3).

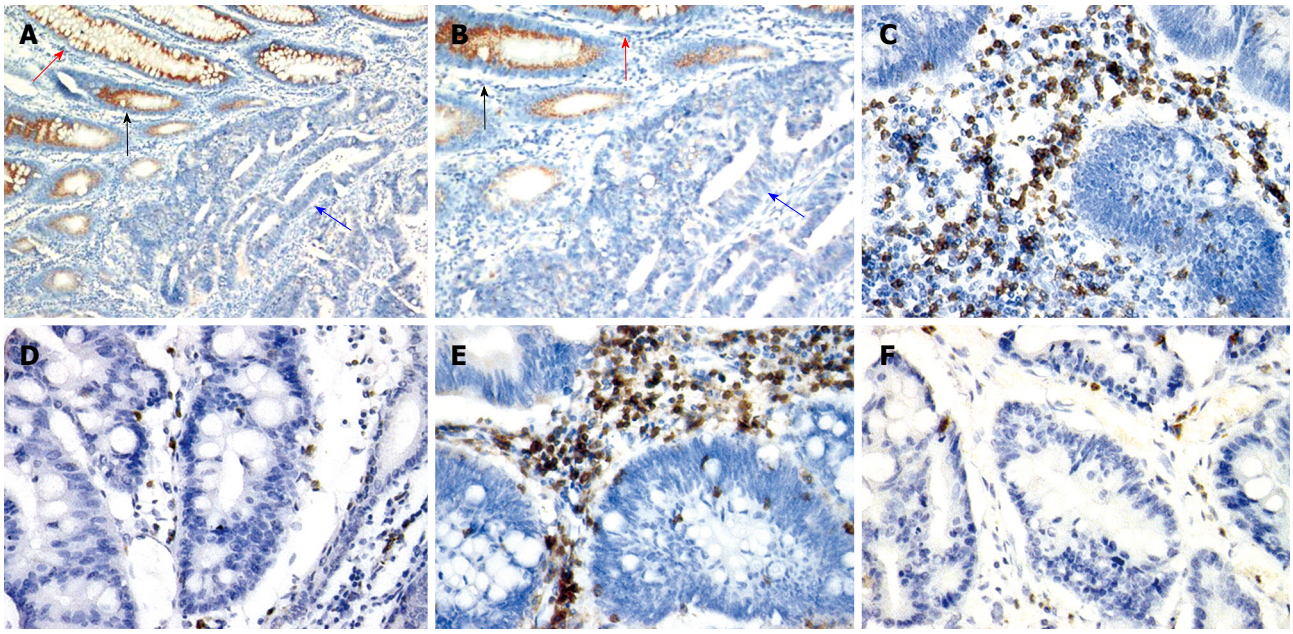


Figure 1 Expression level of CRT in cancer nest (A) and in its adjacent normal epithelium (B) [× 100 in A, × 200 in B, normal epithelium (red arrow), atypical hyperplasia (black arrow) and tumor tissue (blue arrow), respectively], high infiltration of CD3+ cells (C) and CD45RO+ cells (E) in colon cancer stroma (× 400), low infiltration of CD3+ cells (D) and CD45RO+ cells (F) in colon cancer stroma (× 400).

Table 3 Univariate survival analysis (n = 68)

Parameters	Survival time (mo)		*P*
	< 60	⩾ 60	
Gender			0.542
Male	10	28	
Female	6	24	
Age (yr)			0.973
⩾ 60	9	29	
< 60	7	23	
Tumor sites			0.722
Left hemicolon	10	35	
Right hemicolon	6	17	
Pathological grade			0.919
G1	2	8	
G2	12	39	
G3	2	5	
Infiltration of CD3+ cells per high-power field			0.000
> 18	6	45	
⩽ 18	10	7	
Infiltration of CD45RO+ cells per high-power field			0.000
> 26	5	46	
⩽ 26	11	6	
Calreticulin expression			
(-)	10	16	
(+)-(+++)	6	36	0.022

Cox regression model revealed that neither expression of CRT nor infiltration of CD3+ cells or CD45RO+ cells in colon cancer had an independent prognostic value (Table 4).

DISCUSSION

In this study, the expression level of CRT was lower in colon cancer than in its adjacent normal epithelium, indicating that expression of CRT is associated with infiltration of CD45RO+ cells rather than with CD3+ cells and may serve as a mechanism underlying immune escape in colon cancer, although it is not an independent prognostic indicator.

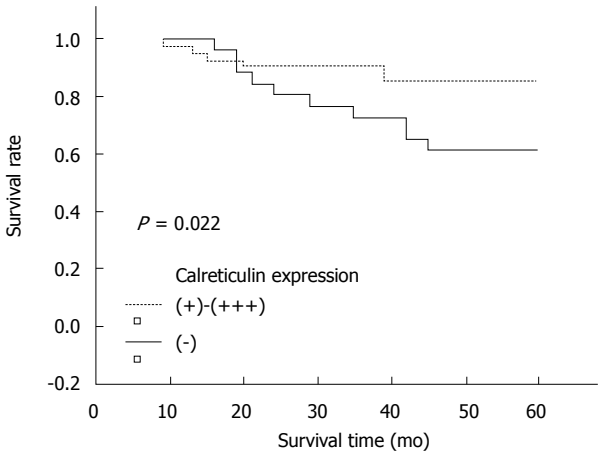


Figure 2 Relation between CRT expression and 5-year survival rate.

A few studies have examined the role of CRT in the progression of colon cancer[40,41]. Toquet *et al*[40] reported that the expression of CRT is decreased in non-mucinous colonic adenocarcinoma while Vougas reported that the expression of CRT is increased in poorly-differentiated colon cancer and advanced tumor[41]. In this study, however, neither the pathological classification nor the differentiation grade was associated with the expression level of CRT.

It is still unknown whether CRT is involved in the mechanism underlying immune escape in colon cancer. Colorectal cancer with microsatellite instability (MSI-H) is immunogenic due to infiltration of a large number of lym-

Table 4 Multivariate survival analysis (n = 68)

Parameters	B	SE	Wald	df	Sig.	Exp(B)	95% CI for Exp (B)	
							Lower	Upper
Pathological grade	0.172	0.261	0.435	1	0.510	1.188	0.712	1.979
Gender	-0.027	0.247	0.012	1	0.912	0.973	0.599	1.580
Age	0.079	0.255	0.095	1	0.758	1.082	0.656	1.784
Tumor site	0.085	0.276	0.094	1	0.759	0.919	0.535	1.579
Infiltration of CD3+ cells	-0.057	0.511	0.013	1	0.911	0.944	0.347	2.537
Infiltration of CD45RO+ cells	-0.743	0.552	1.809	1	0.179	0.476	0.161	1.404
Expression level of calreticulin	0.133	0.279	0.226	1	0.634	0.876	0.507	1.512

B: Regression coefficient; SE: Standard error; df: degree of freedom; Sig.: Significant; Exp (B): Odds ratio; CI: Confidence interval.

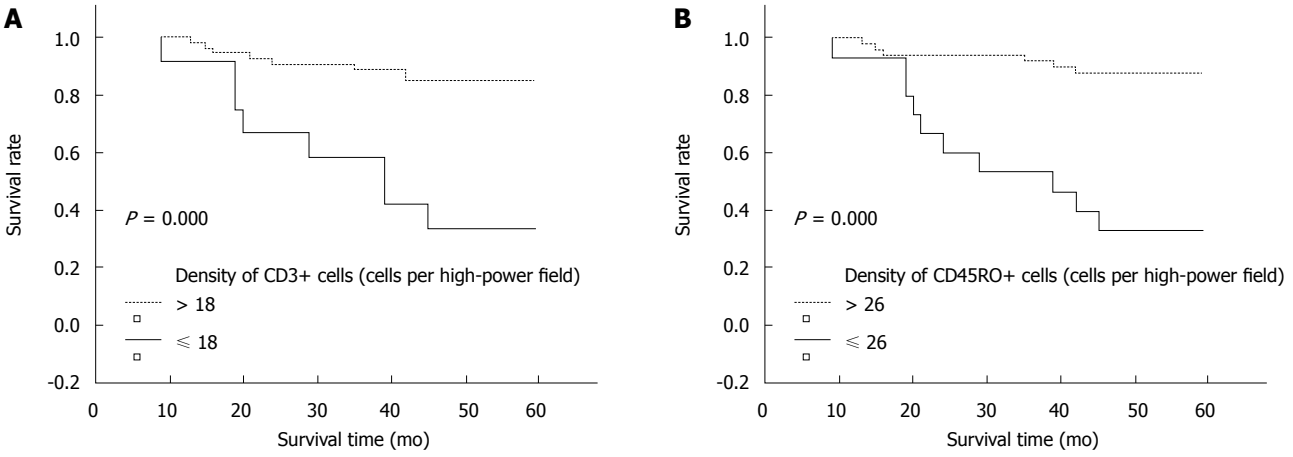


Figure 3 Correlation between high infiltration of CD3+ cells (A) and CD45RO+ cells (B) in colon cancer and 5-year survival rate of patients.

phocytes, resulting in a favorable prognosis[42,43]. Microarray analysis showed that CRT expression was up-regulated in 27 cases of colorectal cancer with MSI-H, while quantitative RT-PCR analysis failed to confirm it in another 26 cases of colorectal cancer with MSI-H[44]. In this study, the relation between expression level of CRT and infiltration of lymphocytes was assessed, which indicates that expression of CRT is related with infiltration of CD45RO+ cells in colon cancer. Since CD45RO+ cells contribute to a favorable prognosis of colon cancer patients, it is reasonable to infer that CRT expression is involved in immune response that occurs in colon cancer. However, this study failed to show that either CRT expression or infiltration of CD45RO+ cells was associated with the 5-year survival rate in a Cox statistical model. Therefore, further study is needed to confirm the role of CRT expression in the progression of colon cancer.

In conclusion, a low expression level of endogenous "danger signals", such as CRT, may represent a new mechanism underlying immune escape in colon cancer.

COMMENTS

Background

There is evidence that colorectal cancer is immunogenic. The mechanisms by which colorectal cancer cells induce an immunologic response remain unknown. A subset of damage-associated molecular patterns (DAMPs) has been recently found to be involved in the induction of immune response. Calreticulin is one of the most important DAMPs. Whether calreticulin is associated with the immunogenicity of colon cancer remains controversial.

Research frontiers

Calreticulin is a multifunctional chaperone protein which mainly locates in the lumen of endoplasmic reticulum (ER). When exposed on the cell surface, calreticulin works as an "eat me" signal for dendritic cells and macrophages, initiating an immune response. Whether the expression of calreticulin in colon cancer is involved in induction of immune response is a hot topic on the role of calreticulin in pathogenesis of colon cancer.

Innovations and breakthroughs

Studies indicate that the expression of calreticulin is decreased in non-mucinous colonic adenocarcinoma while is increased in poorly -differentiated colon cancer and advanced tumor. Other studies have shown that one of the calreticulin fragments inhibits angiogenesis and growth of colon cancer cells. However, the correlation between expression of calreticulin and survival of colon cancer patients remains unknown. In this study, the expression of calreticulin in stage ⅢB colon cancer was associated with the lower infiltration of CD45RO+ lymphocytes which was associated with a shorter 5-year survival time, suggesting that reduced expression of CRT may serve as a mechanism underlying immune escape in colon cancer.

Applications

In this study, reduced expression of endogenous "danger signals", such as calreticulin, was found to be a new mechanism underlying immune escape in colon cancer, which is important for a better understanding of the immunogenicity of colon cancer, thus providing a new immunotherapy for colon cancer.

Terminology

Calreticulin: A highly conserved 46 kDa Ca^{2+}-binding protein, which is mainly located in the lumen of ER and has various versatile functions, like chaperone activity, regulation of Ca^{2+} homeostasis, and adhesion signaling. When cells were treated with anthracyclines, oxaliplatin, radiation and hypoxia, calreticulin exposure works as an "eat me" signal for dendritic cells and macrophages, initiating an immune response.

Peer review

This is a well-conducted study on the correlation between calreticulin expression and infiltration of lymphocytes in colon cancer and their impact on the 5-year survival time of patients with stage ⅢB colonic cancer.

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BRIEF ARTICLE

Early mortality of alcoholic hepatitis: A review of data from placebo-controlled clinical trials

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Abstract

AIM: To investigate the early mortality of placebo-treated alcoholic hepatitis patients.

METHODS: Mortality data about alcoholic hepatitis patients who participated in randomized placebo-controlled trials were searched from PubMed, EMBASE, and Cochrane Library, extracted and analyzed.

RESULTS: A total of 661 placebo-treated patients in 19 trials were included. The overall mortality rate was 34.19% with a median observation time of 160 d (range 21-720 d). Hepatic failure, gastrointestinal bleeding and infection were the three main causes of death, accounting for 55.47%, 21.17% and 7.30% of all deaths, respectively. One-month mortality data about 324 placebo-treated alcoholic hepatitis patients in 10 trials were reported with a pooled mortality rate of 20.37%. The one-month mortality rate of patients with moderate to severe alcoholic hepatitis tended to be higher than that of general patients (22.69% *vs* 10.93%, $P < 0.05$), whereas no significant difference was observed between the patients from North America or Europe (22.43% *vs* 18.45%, $P > 0.05$), neither any difference was found between the studies published before and after 1990 (18.18% *vs* 21.88%, $P > 0.05$).

CONCLUSION: Alcoholic hepatitis is a severe liver disease with a high mortality rate, and hepatic failure, gastrointestinal bleeding and infection are the three main causes of death.

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Key words: Alcoholic hepatitis; Mortality; Placebo

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INTRODUCTION

Alcohol abuse causes a variety of liver diseases including alcoholic steatosis, alcoholic hepatitis, liver fibrosis and cirrhosis[1,2]. Generally, alcoholic steatosis is a benign lesion with a favorable prognosis if the patient abstains from alcohol use, whereas alcoholic hepatitis, which is observed in approximately 20% of heavy drinkers, is much more serious and requires treatment[3]. With increasing alcohol consumption worldwide, alcoholic liver disease has become a significant global health concern[4,5].

Alcoholic hepatitis is characterized by the development of hepatocellular necrosis and inflammation in

alcoholic patients[6]. Its pathogenesis is a multifactorial process involving metabolism of alcohol to toxic products, Kupffer cell stimulation by endotoxin and nutritional impairment lead to liver injury and inflammation[7]. The treatment of alcoholic hepatitis is still mostly symptomatic or empirical at best. Abstinence and supportive care are the two critical principles for the treatment of alcoholic hepatitis[7]. Several clinical trials have shown that corticosteroids can improve the short-term survival of patients with severe alcoholic hepatitis[8]. Recent evidence also suggests that inhibiting tumor necrosis factor-alpha release is beneficial for alcoholic hepatitis[9].

Since most studies on alcoholic hepatitis have focused either on its pathogenesis or on its potential therapies[7-9], its natural history has not been clearly defined. One possible explanation for this is that many alcoholic hepatitis patients are treated with available therapies immediately upon its diagnosis. Active therapies may significantly influence its natural progression and it is inappropriate to withdraw the treatment for studying its natural history. An understanding of the progression of alcoholic hepatitis would contribute to its prevention and treatment.

Randomized placebo-controlled clinical trials are considered the gold standard for evaluating the efficacy of medical interventions on the disease[10]. Data from placebo-controlled trials may also provide valuable information about its natural history. Since the use of placebo has very little effect on the progression of alcoholic hepatitis[11], placebo-treated patients may be the most suitable subjects for studying its natural history. Since a number of randomized placebo-controlled clinical trials are available on evaluating the treatment of alcoholic hepatitis worldwide[9], it has become feasible to study its natural history by extracting data from these studies.

In this study, we conducted a pooled analysis of data about alcoholic hepatitis patients who were treated with placebo in the eligible randomized placebo-controlled trials, in an attempt to evaluate the early mortality of such patients.

MATERIALS AND METHODS

Systematic literature search

English literature on alcoholic hepatitis patients in randomized placebo-controlled trials was searched from PubMed (1966 - March 2009), EMbase (1974 - March 2009), and Cochrane Library (2009, Issue 1) by two independent investigators using the search terms "alcoholic hepatitis", "alcoholic steatohepatitis", "alcoholic liver disease", "placebo" and "randomized controlled trial". Editorial or letter or comment or review was excluded. Reference lists of the retrieved relevant articles were also searched for additional trials.

Study selection

Two investigators independently performed the study selection. Search findings were screened for potentially eligible trials and full-text articles were obtained for their detail evaluation. Disagreements between the two investigators regarding studies included were solved by consensus.

Studies fulfilling the following criteria were included: studies of a randomized placebo-controlled clinical trial in alcoholic hepatitis patients, studies with enrollment, treatment and analysis of patients well-defined, and studies with more than 15 patients involved in the placebo arm, since we believed that they would not generate enough meaningful data to allow quality assessment. Trials were excluded if relevant data were not extractable or if the case-mix included unclassified alcoholic liver disease patients. Alcoholic hepatitis patients in the placebo arms that received basic active treatment with drugs such as steroids were also excluded.

Data extraction and quality assessment

Data extracted from the studies included name of the first author, publication year, study design, location(s), sample size, randomized method, and gender, mean age and disease severity of patients, description of active therapy, duration of therapy, duration of follow-up, mortality data and causes of death. Data extraction was performed by two independent reviewers. Sections of METHODS and RESULTS were coded to blind reviewers to the above information. Primary investigators were contacted if data were incomplete. The methodological quality of studies included was assessed using a validated quality checklist[12] with a maximum score of 32. A score of 12 (38%) or greater was considered to have acceptable quality[13].

Statistical analysis

Pooled estimate of each variable of interest was calculated and presented as a mean. The cumulative rate of each outcome of interest was calculated in the placebo arm of eligible studies. χ^2 test was used to compare qualitative variables. $P < 0.05$ (2-tailed test) was considered statistically significant.

RESULTS

Description of trials

We identified 892 potentially relevant articles and excluded 845 articles describing studies that obviously did not fulfill the inclusion criteria in this study by reviewing their titles and abstracts. Of the 47 studies selected for full-text review, 19 were excluded because they were not designed as placebo-controlled trials, 3 were excluded because the patients received other active medications[14-16], 4 were excluded because less than 15 patients received placebo[17-20], 1 was excluded because detailed survival data were not available[21], and 1[22] sharing the same placebo with a previous patient[23] was also excluded. Thus, only 19 studies fulfilled the inclusion criteria in this study (Figure 1).

Of these 19 clinical trials, 9 were single center studies, 10 were multi-center studies. A total of 661 patients with a mean age of 46.4 years served as placebo controls. Detailed information and summarized information about the studies included are shown in Tables 1 and 2, respectively.

Pooled mortality

Of the 661 patients, 226 (34.19%) died during a median

Table 1 Description of studies included in pooled analysis

Study	Country	Study type	Severity of alcoholic hepatitis	Placebo size	Mean age	Gender (M/F)	Active therapy	Therapeutic time	Follow-up time	Death (1-mo/ overall)
Helman *et al*[24], 1971	US	Single center	Unclassified	17	47.7	NA	Prednisolone	4 wk	3 mo	NA/6
Blitzer *et al*[25], 1977	US	Single center	Unclassified	16	48.4	NA	Prednisolone	26 d	9 wk	NA/5
Maddrey *et al*[26], 1978	US	Single center	Moderate to severe	31	42.3	23/8	Prednisolone	28-32 d	4 wk	6/6
Baker *et al*[27], 1981	US	Single center	Unclassified	25	41.0	13/12	Insulin/glucagon	3 wk	3 wk	NA/6
Hallé *et al*[28], 1982	US	Single center	Severe	36	38.9	32/4	Propylthiouracil	6 wk	8 wk	7/7
Mendenhall *et al*[29], 1984	US	Multi-center	Moderate to severe	88	50.4	NA	Oxandrolone and prednisolone	1 mo	2 yr	NA/50
Fehér *et al*[30], 1987	Hungary and Spain	Multi-center	Unclassified	33	46.0	18/15	Insulin/glucagon	3 wk	3 wk	NA/14
Carithers *et al*[31], 1989	US	Multi-center	Severe	31	44.4	21/10	Methylprednisolone	4 wk	4 wk	11/11
Trinchet *et al*[32], 1989	Belgique	Multi-center	Unclassified	34	52.0	17/17	Colchicine	6 mo	6 mo	0/0
Akriviadis *et al*[33], 1990	US	Single center	Severe	36	40.8	25/11	Colchicine	1 mo	4 mo	6/8
Panos *et al*[34], 1990	UK	Single center	Unclassified	51	49.3	22/29	Polyunsaturated phosphatidyl choline	2 yr	2 yr	NA/20
Bird *et al*[35], 1991	UK	Single center	Severe	43	51.0	16/14	Insulin/glucagon	3 wk	6 mo	14/15
Mezey *et al*[36], 1991	Spain	Multi-center	Severe	26	43.7	12/14	Amino acid suppl.	3 wk	23 mo	5/16
Ramond *et al*[23], 1992	France	Multi-center	Severe	29	48.2	9/20	Prednisolone	4 wk	6 mo	NA/16
Trinchet *et al*[37], 1992	France	Multi-center	Severe	35	48.0	17/18	Insulin/glucagon	3 wk	1 mo	5/5
Bird *et al*[38], 1998	UK	Multi-center	Unclassified	30	51.0	16/14	Amlodipine	4 wk	4 wk	7/7
Akriviadis *et al*[9], 2000	US	Single center	Severe	52	40.8	40/17	Pentoxifylline	4 wk	160 d	NA/24
Mezey *et al*[39], 2004	US and Spain	Multi-center	Unclassified	26	49.0	16/10	Vitamin E	3 mo	1 yr	NA/5
Boetticher *et al*[40], 2008	US	Multi-center	Moderate to severe	22	49.1	17/5	Etanercept	3 wk	6 mo	5/5

NA: Not available.

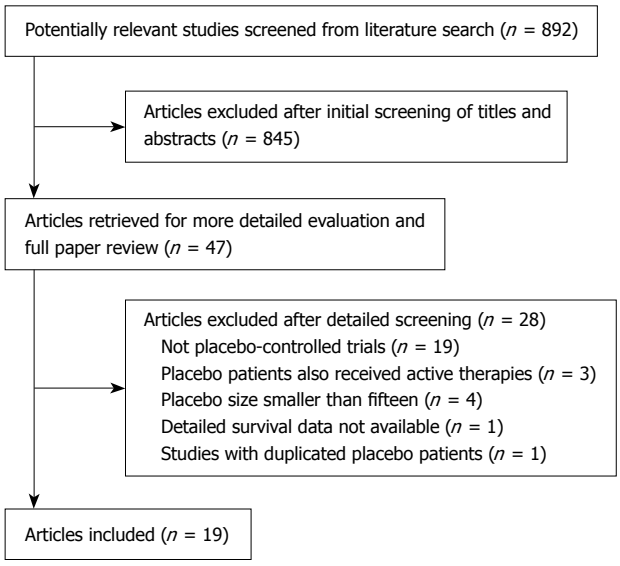


Figure 1 Schema for literature search and study inclusion.

observation time of 160 d (range 21-720 d). Detailed causes of 137 deaths were available from 13 of the studies. Hepatic failure, gastrointestinal bleeding and sepsis were the three main causes of death, accounting for 55.47%, 21.17% and 7.30% of all deaths, respectively. One-month mortality data were available in 10 studies and the pooled mortality rate for these patients was 20.37% (66/324).

Subgroup analysis

Since the observation time was different among the studies included, we did not directly compare the overall mortality rate of alcoholic patients. In subgroup analysis, we compared the one-month mortality rate of alcoholic patients based on the data extracted from 10 studies.

Of the 10 studies selected for comparison, 2 were performed in moderate to severe alcoholic hepatitis patients, 6 in severe alcoholic hepatitis patients, 2 in unclassified alcoholic hepatitis patients. The pooled one-month mortality rate of such patients tended to increase with increasing severity of the disease. The pooled mortality rate of unclassified, moderate to severe and severe alcoholic hepatitis patients was 10.94% (7/64), 20.75% (11/53), and 23.19% (48/207), respectively (Table 2).

Of the 10 studies, 5 were conducted in the United States and 5 in Europe (UK, France, Belgium and Spain) showing a pooled mortality rate of 22.44% (35/156) and 18.45% (31/168), respectively. No significant difference was found in pooled mortality rate between the patients from North America or Europe ($\chi^2 = 0.791$, $P = 0.409$) (Table 2).

Of the 10 studies, 5 were published before December 31, 1990, showing a pooled total mortality rate of 17.86% (30/168), 5 were published after January 1, 1991, showing a pooled mortality rate of 23.08% (36/156). No significant difference was observed in pooled mortality rate between the publication time ($\chi^2 = 1.359$, $P = 0.244$) (Table 2).

DISCUSSION

In this study, we extracted data about alcoholic hepatitis patients in randomized placebo-controlled trials and

Table 2 Comparison of pooled one-month mortality by different variables

	Studies (*n*)	Placebo-treated patients (*n*)	Gender (M/F)	One-month death (*n*)	One-month mortality rate (%)	χ^2	*P* value
Severity of disease							
Unclassified	2	64	33/31	7	10.94	4.375	0.038
Moderate to severe	8	260	171/89	59	22.69		
Geographical area(s)							
US	5	156	119/37	35	22.44	0.791	0.409
Europe	5	168	85/83	31	18.45		
Publication year							
Before 1990	5	168	118/50	30	17.86	1.359	0.271
After 1991	5	156	86/70	36	23.08		

analyzed their mortality, showing that the mortality rate of alcoholic hepatitis patients is positively correlated with the severity of the disease. Our pooled analysis also showed that hepatic failure, gastrointestinal bleeding and infection were the three main causes of early death of alcoholic hepatitis patients, which may be of great importance in clinical practice.

Unlike nonalcoholic hepatitis, a chronic form of liver disease associated with obesity, alcoholic hepatitis is an acute and potentially life-threatening form of liver disease[41]. The cause of its early mortality is not clear since it is difficult to evaluate a sufficient number of such patients without prompt treatment upon diagnosis. In this study, the data about 661 placebo-treated patients extracted from 19 placebo-controlled trials were evaluated, showing that the mortality rate of alcohol hepatitis patients is 34.19% with a pooled one-month mortality rate of 20.37%, which increases with increasing disease severity. These findings suggest that alcoholic hepatitis patients should be treated promptly upon diagnosis.

Recognizing the causes of death in alcoholic hepatitis patients contributes to the treatment of alcoholic hepatitis. Hepatic failure, gastrointestinal bleeding and infection are the three main causes of early death in alcoholic hepatitis patients. Hepatic failure can be prevented by avoiding exposure to certain hepatotoxic drugs, such as acetaminophen, carbon tetrachloride and galactosamine. Corticosteroid therapy is effective against alcoholic hepatitis[8], but it may result in complications such as gastrointestinal bleeding. It is, therefore, necessary to evaluate the risks and benefits of corticosteroid therapy for alcoholic hepatitis before it is used.

Due to the limited available data, there are some limitations in this study. First, we did not analyze the factors predicating the early mortality rate of alcoholic hepatitis patients since necessary data could not be extracted from the studies included. However, our pooled analysis provided the main causes of death, which may be of importance in clinical practice. Second, we did not clarify whether the mortality rate of alcoholic hepatitis patients varies between males and females. Since females are reported to be more sensitive to alcohol abuse[7], it would be of interest to compare mortality rates of both genders. Third, we did not analyze the potential heterogeneity due to the relative small number of placebo patients. We grouped all the patients irrespective of their age, gender, ethnic distinction, or severity of disease. The pooled analysis of all these cases provided a clearer general picture of early mortality rate in a relatively large sample of alcoholic hepatitis patients.

In summary, alcoholic hepatitis is a severe liver disease with a high early mortality, especially among those with moderate to severe alcoholic hepatitis. Hepatic failure, gastrointestinal bleeding and infection are the three main causes of early death in alcoholic hepatitis patients.

COMMENTS

Background

Alcoholic hepatitis is a potentially life-threatening complication of alcohol use, but its natural history has not been clarified so far.

Research frontiers

This study investigated the early mortality of placebo-treated alcoholic hepatitis patients.

Innovations and breakthroughs

This study has confirmed that alcoholic hepatitis is a severe liver disease with a high early mortality, especially among those with moderate to severe disease.

Applications

The results of this study suggest that hepatic failure, gastrointestinal bleeding and infection should be treated in order to prevent early death of alcoholic hepatitis patients.

Terminology

Alcoholic hepatitis is a disease resulting from hepatocellular necrosis and inflammation in alcoholic patients. Its pathogenesis is a multifactorial process involving metabolism of alcohol to toxic products. Kupffer cell stimulation by endotoxin and nutritional impairment lead to liver injury and inflammation, *etc*.

Peer review

This is an interesting review of data concerning the early mortality of alcoholic hepatitis. The authors critically discussed the limitation of their survey.

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CASE REPORT

Acute respiratory distress syndrome associated with severe ulcerative colitis

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Abstract

Various extraintestinal manifestations including pulmonary abnormalities have been reported in patients with ulcerative colitis. Acute respiratory distress syndrome (ARDS) is a serious and fatal pulmonary manifestation. We have experienced a 67-year-old male patient with ARDS associated with a severe type of ulcerative colitis (UC). Severe dyspnea symptoms occurred during the treatment of UC in a previous hospital and the patient was transferred to our hospital on June 27, 2007. Both blood and sputa cultures for bacteria and fungi were negative. Cytomegalovirus antigenemia was also not detected. From the clinical and radiological [Chest X-ray, computed tomography (CT)] findings, the patient was diagnosed with ARDS on the basis of the definition of ARDS developed by the European-American Consensus Conference on ARDS. Both colonic inflammations and ARDS symptoms of the patient were resistant to any medical treatment including corticosteroids and antibiotics. However, ARDS symptoms were dramatically improved after surgical colectomy. We believe that severe colonic inflammation from UC was closely associated with the onset of ARDS of the patient. Our case report suggests that a severe type of ulcerative colitis might be taken into consideration as one of the predisposing factors of ARDS.

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Key words: Acute respiratory distress syndrome; Ulcerative colitis; Inflammatory bowel disease; Extraintestinal manifestation

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INTRODUCTION

Ulcerative colitis (UC) is a chronic intractable colitis with unknown etiology. Various extraintestinal manifestations, including hepatobiliary, musculoskeletal, dermatologic, ocular and pulmonary symptoms, have been reported in patients with UC[1-3]. Acute respiratory distress syndrome (ARDS) is a serious and fatal pulmonary manifestation and overall mortality from ARDS has been typically reported to be greater than 50%[4]. Here, we showed an ARDS patient associated with severe UC. ARDS symptoms of the patient were resistant to any medical treatment but dramatically improved after surgical colectomy. Our case suggests that severe colonic inflammation from UC was closely associated with the onset of ARDS.

CASE REPORT

A 67-year-old male patient visited a previous hospital due to bloody diarrhea on March 1, 2007. He was diagnosed as having UC based on clinical, endoscopic and histological criteria. He was admitted to the hospital and underwent treatment with mesalamine, prednisone (40 mg), and azathioprine (50 mg). Leukocytapheresis therapy was also performed. However, his abdominal symptoms and endoscopic findings got worse and an intensive intravenous steroid regimen with 60 mg prednisone was started. During the treatment, he complained of severe dyspnea and a chest-X ray revealed pulmonary edema. Treatment with azathioprine and mesalamine was discontinued after he complained of severe dyspnea. Then, the patient was transferred to Akita University Hospital for intensive treatment on June 27, 2007. On physical examinations, he had tachycardia (134 bpm) and a fever (38.1℃), and coarse crackles were audible in both lung fields. Tenderness was observed in the lower abdomen but no symptoms suggesting peritonitis were found. Blood chemistry values on June 28 were as follows: hemoglobin (Hb) 8.9 g/dL, hematocrit (Ht) 27.9%, white blood cell count (WBC) 3100/L, C-reactive protein (CRP) 2.92 mg/dL, total protein (TP) 4.4 g/dL, albumin 2.4 g/dL, L-aspartate 2-oxoglutarate aminotransferase (AST) 74 U/L, L-alanine 2-oxoglutarate aminotransferase (ALT) 73 U/L, alkaline phosphatase (ALP) 785 U/L, blood urea nitrogen (BUN) 31.9 mg/dL, creatinine (Cr) 0.38 mg/dL. Anemia, inflammatory changes, hypoproteinemia, and liver dysfunction were observed. Chest X-ray showed pulmonary edema without enlargement of the heart (Figure 1A). Arterial blood gas values were PaO_2 39 mmHg; $PaCO_2$ 35.7 mmHg; pH 7.518; and HCO_3 28.4 mEq/L, showing impaired oxygenation. Ultrasound cardiography showed normal heart function, with no enlargement of the right atrium and ventricle. From these clinical and radiological findings, the patient was diagnosed as having ARDS. Chest computed tomography (CT) scan images of the patient were also compatible with ARDS (Figure 2). Both blood and sputum cultures for bacteria and fungi taken in the hospital were negative. Low levels of Candida antigen and a slight increase of β-D glucan were found in the patient's blood. However, serum Candida antigen promptly decreased after treatment with an antifungal drug (fosfluconazole). Cytomegalovirus antigenemia was not detected. Dyspnea of the patient was severe and not improved by nasal oxygen supplementation. Then, the patient was admitted to the intensive care unit (ICU) with intubation and positive pressure ventilation. However, despite intensive medical treatment including prednisone, antibiotics (cefazolin sodium, imipenem/cilastatin sodium), and sivelestat sodium hydrate, pulmonary condition and abdominal symptoms of the patient were not improved. Colonoscopic examination on July 2nd also showed severe colonic inflammation with multiple deep ulcers (Figure 3). However, cytomegalovirus antigenemia was not detected. Both fecal culture and fecal Clostridium difficile toxins were negative. Bronchoscopy on July 6th showed wet bronchial mucosa with a little inflammatory change, and no bacteria and fungi were detected by culturing the sputa collected by bronchoscopy. Colonoscopic findings together with his clinical course strongly suggested that the predisposing factor of ARDS of the patient might be severe inflammation of the colon. Thus, we thought that surgical colectomy was essential to rescue the patient from ARDS, and subtotal colectomy was performed on July 11. Postoperatively, gas exchange was dramatically improved, and he was weaned off the ventilator on July 19. A chest X-ray taken August 11 (Figure 1B) was also significantly improved. After an additional 55 d recovery, he was discharged from the hospital with no respiratory symptoms.

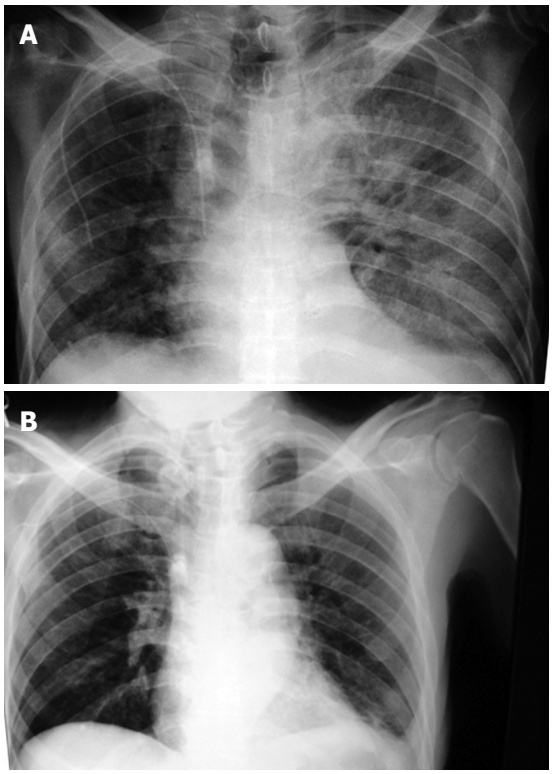


Figure 1 Chest X-ray. A: Chest X-ray taken at time of admission showing Acute respiratory distress syndrome (ARDS); B: Chest X-ray after surgical colectomy showing a significant improvement of ARDS.

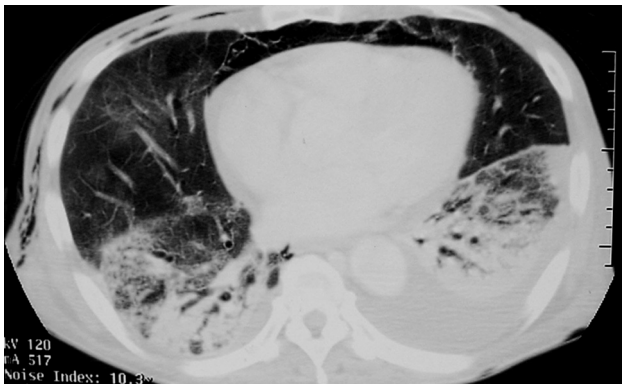


Figure 2 Chest computed tomography (CT) scan image taken before surgical treatment showing compatibility with ARDS.

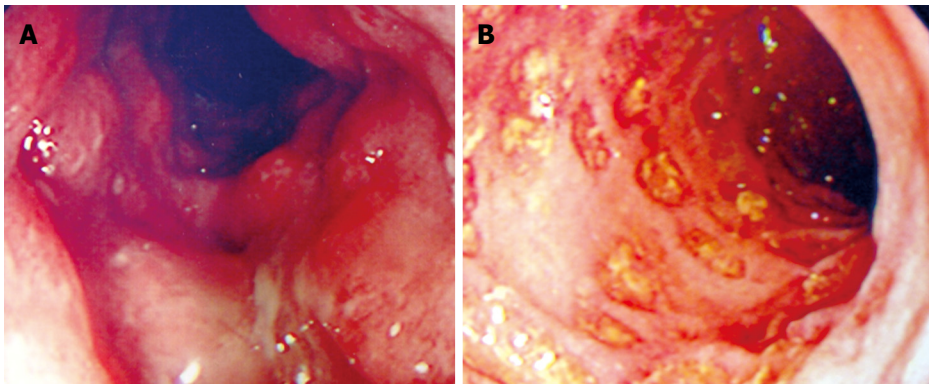


Figure 3 Colonoscopic images showing deep ulcer with severe mucosal edema (A) and multiple discrete ulcers (B).

DISCUSSION

Both UC and Crohn's disease are chronic intractable inflammatory bowel diseases (IBD). It has been shown that 30%-50% of IBD patients have pulmonary abnormalities[1]. In addition, drug-induced lung injuries have also been reported in IBD patients[5,6]. ARDS is a complex response of the lung to direct and indirect insults and numerous predisposing factors causing ARDS, such as toxic inhalation, diffuse infection, sepsis, pancreatitis, *etc*., have been reported[4]. With regard to an association between UC and ARDS, only one ARDS patient accompanied by UC has been reported to date[7]. In this report, we diagnosed the patient as having ARDS based on the definition of ARDS developed by the European-American Consensus Conference on ARDS[4], namely, $PaO_2/FiO_2 < 200$, the detection of bilateral pulmonary infiltrates on frontal chest radiography, and no clinical evidence of elevated left atrial pressure. With regard to the predisposing factors of ARDS, we could not detect particular factor that clearly caused ARDS in our patient. Although very low levels of Candida antigen was detected in the blood, Candida was not detected by sputa cultures and ARDS symptoms of the patient were not improved after the decrease in the Candida antigen. Thus, we did not think that Candida was the predisposing factor of ARDS in our patient. Other fungi as well as Candida were not detected by sputa cultures, which were examined several times. Although severe inflammation with deep ulcers was endoscopically observed in the colonic mucosa, Cytomegalovirus antigenemia was not detected in the patient during the admission.

Nagy *et al*[6] recently reported a case of interstitial pneumonitis, which was suggested to be ARDS, in a patient with UC treated with azathioprine (150 mg/d, 10 years). They demonstrated that the pulmonary condition of this patient was gradually improved by the discontinuation of azathioprine and treatment with corticosteroids. Our patient was also treated with azathioprine. However, the dose of azathioprine of our patient (50 mg/d, 2 mo) was much smaller than that of Nagy *et al*'s case[6]. Although an association between azathioprine and the onset of ARDS of our patient was not completely excluded, we consider that ARDS symptoms of our patient were not due to administration of azathioprine but due to severe inflammation of ulcerative colitis on the basis of the following findings. First, ARDS symptoms were not improved but worsened after the discontinuation of azathioprine. In addition, ARDS symptoms were not improved by the intravenous corticosteroid treatment. Second, ARDS symptoms of the patient appeared to be correlated with the severity of UC and were dramatically improved after surgical colectomy. From these findings, we believe that severe colonic inflammation from UC was closely associated with the onset and the persistence of ARDS of our patient. It is suggested that pathogenic factors that caused ARDS in our patient were probably produced in the inflamed colon. ARDS is a serious and fatal pulmonary manifestation caused by a systemic inflammatory status. Our case report suggests that a severe type of UC might be taken into consideration as one of the predisposing factors of ARDS.

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CASE REPORT

Filiform polyposis in the sigmoid colon: A case series

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Abstract

Filiform polyposis is a rare condition of uncertain pathogenesis that is usually found in association with Crohn's disease, ulcerative colitis, intestinal tuberculosis or histiocytosis X. We report seven interesting cases of polyposis with various pathologic components, mainly located in the left side of the colon with no associated inflammatory bowel disease, intestinal tuberculosis or histiocytosis X. Multiple finger-like polypoid lesions with the appearance of stalactites were noted on the left side of the colon, especially in the sigmoid area, at the time of colonoscopy. The polyps had a variety of sizes and shapes and were shown to have various histopathologic components among the different patients. Although filiform polyposis localized in the sigmoid colon appears not to have high oncogenic potential, periodic follow-up seems to be needed.

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Key words: Filiform polyposis; Sigmoid colon; Inflammatory bowel disease

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INTRODUCTION

Filiform polyposis is a rare form of pseudopolyposis associated with ulcerative colitis, Crohn's disease, or granulomatous disease, which is formed by non-specific mucosal and submucosal reactions to previous severe inflammation[1-5]. The transverse and descending colon are the most common locations, although the polyps can be seen in any portion of the large bowel[3-6]. However, reports regarding filiform polyposis confined to the sigmoid colon, and the natural history or management of incidentally detected filiform polyposis in patients without the definite history of ulcerative colitis, Crohn's disease, or intestinal tuberculosis are inadequate[7,8].

We report seven interesting cases of filiform polyposis with mixed histologic components, including mainly inflammatory and hyperplastic or adenomatous components, located in the sigmoid area, without a definite history of severe inflammation such as inflammatory bowel disease (IBD) or intestinal tuberculosis.

CASE REPORT

Case 1

A 38-year-old male patient had sudden onset abdominal pain, diarrhea, and hematochezia for 2 d and had been diagnosed as having infectious colitis. He did not have a medical history of tuberculosis, IBD, or infectious colitis. Colonoscopic examination showed multiple, variable-sized polyps that were found in the sigmoid colon (Figure 1A). These polyps were histopathologically proven to be inflam-

matory and hyperplastic. Up to 44 mo later, there was no evidence of malignant change in the polyposis on follow-up colonoscopy examination.

Case 2

A 64-year-old healthy male patient had no medical history of IBD or lower gastrointestinal symptoms. Colonoscopy was performed for screening purposes. On the colonoscopic examination, multiple long worm-like polyps were noted at the sigmoid colon (Figure 1B). These growths were histopathologically proven to be inflammatory polyps. Two large polyps were also found in the ascending colon and snare polypectomy was performed. These polyps were adenomas by pathologic evaluation.

On the follow-up colonoscopic examination 6 mo later, multiple finger-like polypoid lesions with the appearance of stalactites persisted on the left side of the colon, especially in the sigmoid area (Figure 1C). Large polyps with a hyperemic mucosa were removed by snare. These polyps were hyperplastic, tubular adenomas and filiform polyps with lymphoid hyperplasia. Up to 43 mo later, there was no evidence of malignant change in the polyposis on follow-up colonoscopy examination.

Case 3

A 37-year-old male patient had frequent intermittent diarrhea after abdominal pain and was diagnosed as having irritable bowel syndrome. Colonoscopy was performed and multiple polyps were seen, which were densely packed, forming a roof in the sigmoid colon (Figure 1D). A representative polyp was removed by snare and shown to be a filiform or hyperplastic polyp on pathologic assessment. Up to 46 mo later, there was no evidence of malignant change in the polyposis on follow-up colonoscopy examination.

Case 4

A 67-year-old asymptomatic male patient stated that colonofiberscopy had been performed at another hospital 2 years previously and the colonoscopic findings were lesions suspicious for intestinal tuberculosis. Colonofiberscopy was conducted again and multiple polypoid lesions with variable shape and size were found at the sigmoid colon (Figure 1E). To establish the exact pathologic diagnosis, snare polypectomy was performed. The pathologic results revealed that the polyps were mainly filiform and hyperplastic. Up to 46 mo later, there was no evidence of malignant change in the polyposis on follow-up colonoscopy examination.

Case 5

A 77-year-old male patient generally had no lower gastrointestinal symptoms. The colonoscopic findings showed multiple polyps forming a densely packed bridge in the sigmoid colon (Figure 1F). These polyps were pathologically diagnosed as being mainly filiform and hyperplastic. Up to 34 mo later, there was no evidence of malignant change in the polyposis on follow-up colonoscopy examination.

Case 6

An 81-year-old female patient was referred to our hospital for evaluation of sigmoid polyposis. Colonoscopy was performed and worm-like polyps located in the sigmoid colon were shown (Figure 1G). In addition, one polyp was reported as chronic non-specific inflammation with crypt cell hyperplasia and another was a hyperplastic polyp with focal atypical glands, favoring a reactive hyperplasia. Up to 38 mo later, there was no evidence of malignant change in the polyposis on follow-up colonoscopy examination.

Case 7

A 63-year-old female had a history of diarrhea, abdominal pain, and weight loss for several months. The patient was referred to our hospital for further evaluation of multiple colon polyps. On colonoscopy, multiple variable polyps were noted in the cecum, ascending colon, and mainly in the sigmoid colon. Multiple finger- or worm-like polypoid lesions were noted in the sigmoid colon (Figure 1H and I). The polyps were of various size and shape. The tips of the polyps adhered to other parts of the mucosa, forming loops or bridges between opposing walls. Large polyps were removed by snare. All these polyps, except one, were shown to be hyperplastic or filiform (inflammatory) based on pathologic evaluation (Figure 2A and B). Only one polyp was shown to be a villous adenoma (Figure 2C). Up to 36 mo later, there was no evidence of malignant change in the polyposis on follow-up colonoscopy examination. We recommended periodic colonoscopy for cancer surveillance.

DISCUSSION

We report here seven cases of previously undiscovered polyposis in the sigmoid colon without a definite history of severe chronic inflammation including IBD or intestinal tuberculosis. This report is intended to characterize the clinical and pathologic features of filiform polyposis mainly located in the sigmoid colon. The clinicopathologic characteristics of filiform polyposis in the sigmoid colon of all the seven cases are shown in Table 1.

Filiform polyposis is an uncommon entity that is most often encountered in the colon of patients with a history of IBD[2]. Filiform polyposis is characterized by a large number of worm-like polyps lined by histologically normal colonic mucosa[5,8]. Filiform polyps usually have a thin, straight shape resembling the stalks of polyps without the heads[4]. The polyps can range in size from 1.5-3.0 cm in length and up to 0.5 cm in diameter[2,3]. The projections can occur as solitary polyps or as diffuse polyposis distributed over large areas of the colonic mucosa[8]. Long-term inflammation of the colonic mucosa during chronic IBD with alternating periods of ulceration and healing may lead to the formation of finger-like projections, these so-called filiform polyps[2-4]. Only rare cases without a history or evidence of IBD have been reported[3]. Additionally, in rare cases, several

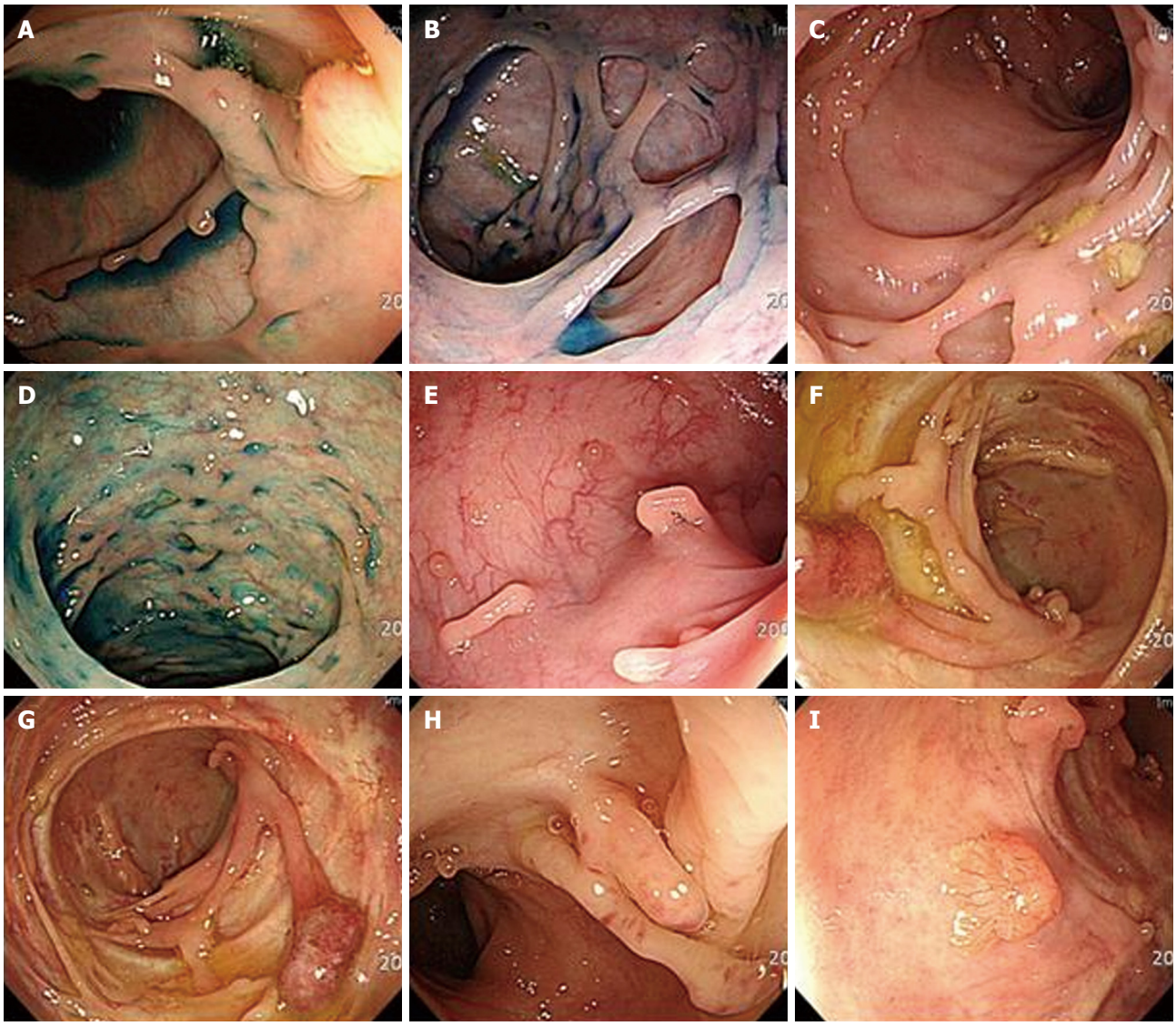


Figure 1 Colonoscopic findings show multiple worm-like or finger-like polypoid lesions, with a stalactite appearance, in the left-sided colon, especially in the sigmoid area (A-I).

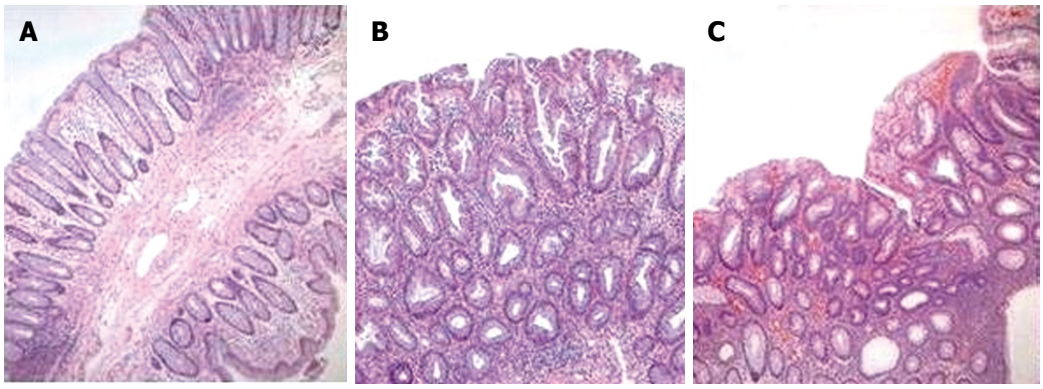


Figure 2 Pathologic features of inflammatory polyp, hyperplastic polyp and tubular adenoma (HE, × 100). A: Inflammatory polyp showing chronic inflammatory cells in lamina propria without hyperplastic or adenomatous epithelial change; B: Hyperplastic polyp showing hyperplastic epithelia with stellate lumen; C: Tubular adenoma showing low grade dysplasia.

filiform polyps form large tumor masses, termed giant filiform polyposis[5]. Histologically, the polyps are filiform, with a central core, containing vessels and smooth muscle fibers[6]. Clinicopathologic and immunopheno-

Table 1 Clinicopathologic characteristics of filiform polyposis in the sigmoid colon

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7
Age (yr)	38	64	37	67	77	81	63
Sex	M	M	M	M	M	F	F
Location	Sigmoid colon	Sigmoid colon	Sigmoid colon	Sigmoid colon	Sigmoid colon	Sigmoid colon	Sigmoid colon
Tuberculosis history	None	None	None	None	None	None	None
IBD	None	None	None	None	None	None	None
Chest PA	None	Normal	Normal	Normal	Normal	Normal	Normal
Pinch biopsy pathologic findings	Inflammatory Hyperplastic	Inflammatory Hyperplastic Tubular adenoma	Hyperplastic	Inflammatory Hyperplastic	Inflammatory Hyperplastic	Inflammatory Hyperplastic	Inflammatory Hyperplastic
Polypectomy pathologic findings	Filiform Hyperplastic	Filiform Hyperplastic Villotubular adenoma	Filiform Hyperplastic	Filiform Hyperplastic	Filiform Hyperplastic	Filiform Hyperplastic	Filiform Villous adenoma
Diarrhea	Yes	None	Yes	None	None	Yes	Yes
Hematochezia	Yes	None	None	None	None	None	Yes
Abdominal pain	Yes	None	Yes	None	None	Yes	Yes
Weight loss	None	None	None	None	None	Yes	Yes
Follow-up period (mo)	44	43	46	46	34	38	36

IBD: Inflammatory bowel disease.

typic studies regarding filiform polyposis without IBD demonstrate that there is generalized polyposis, generally considered to be an asymptomatic sequela of ulcerative colitis[2]. Generally, there is no definite evidence that filiform polyposis itself represents a pre-cancerous condition[2]. Filiform polyposis may resemble villous adenomas on colonoscopy[4]. Therefore, biopsy should be recommended in all cases of filiform polyposis if it is necessary[3,8,9]. Filiform polyposis alone is not an indication for surgical resection, but complications, such as acute massive hemorrhage or intestinal obstruction, may necessitate surgical intervention[10,11].

In our case series it cannot be completely excluded that filiform polyps may have emerged as asymptomatic sequelae of intestinal tuberculosis, IBD, other infectious colitis, ischemic colitis, or histiocytosis X.

Sporadic hyperplastic polyps are generally thought to be non-malignant and malignant transformation into adenocarcinoma has been rarely reported[12]. However, contrary to single sporadic hyperplastic polyps, hyperplastic polyposis is considered to be a pre-cancerous lesion[12-17]. Our case series is distinct from hyperplastic polyposis syndrome. The diagnostic criteria for hyperplastic polyposis generally include the presence of (1) at least 5 histologically diagnosed hyperplastic polyps proximal to the sigmoid colon, of which two are > 10 mm in diameter, or (2) any number of hyperplastic polyps proximal to the sigmoid colon in an individual who has a first degree relative with hyperplastic polyposis, or (3) > 30 hyperplastic polyps of any size that are distributed throughout the colon[12-15]. Our case series did not satisfy these diagnostic criteria.

Diffuse colonic mucosal ulceration is a possible finding in histiocytosis X[18]. Histiocytosis X may have gastrointestinal symptoms such as diarrhea, malabsorption, or gastrointestinal bleeding[18]. However, these gastrointestinal symptoms are rare[18]. Diagnosis of histiocytosis X is based on mucosal biopsy and immunopathologic findings which demonstrate the presence of a disseminated proliferation of histiocytes[18]. Our case series did not satisfy this pathologic finding.

We report seven patients with filiform polyposis mainly localized in the sigmoid colon. Although none of our cases showed progression into adenocarcinoma, various pathologic findings, including mainly filiform polyps, hyperplastic polyps or adenomas, were found. None of the patients had a definite history of IBD, intestinal tuberculosis, infectious or ischemic colitis, but the filiform polyposis seemed to be a sequela of asymptomatic chronic colonic inflammation. Although definite evidence is insufficient, filiform polyposis localized in the sigmoid colon appears not to have high oncogenic potential. However, periodic follow-up seems to be necessary because large polyps were intermittently proven to be adenoma in our case series.

We have reported seven cases of filiform polyposis involving the sigmoid colon in Koreans who had no definite history of IBD or intestinal tuberculosis. Up to the present, studies regarding filiform polyposis syndrome located in the left side of the colon are insufficient. Continuous close follow-up of these seven patients and collection of large numbers of cases should be performed in order to understand the natural history and observe the possibility of progression into cancer.

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Herbal extracts as hepatoprotectants against acetaminophen hepatotoxicity

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Abstract

Many plant-derived natural products have the potential to be hepatoprotective and therefore can be used to treat acute and chronic liver diseases. The challenge is to identify the most promising compounds and evaluate their protective mechanism. In a recently published article, Wang *et al* evaluated extracts of the plant *Gentiana manshurica* Kitagawa (GM) in a model of acetaminophen hepatotoxicity. The authors concluded that GM is hepatoprotective against acetaminophen-induced liver injury due to its antioxidant properties and anti-apoptotic capacity. We would like to discuss the limitations of this experimental approach and question the conclusion based on the data presented in this manuscript and the published literature.

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Key words: Acetaminophen; Drug hepatotoxicity; Herbal extracts; N-acetylcysteine

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TO THE EDITOR

We read with interest a recent paper published in *World Journal of Gastroenterology* by Wang *et al*[1], who showed that 2 h pretreatment with an extract of the plant *Gentiana manshurica* Kitagawa (GM) could protect mice against acetaminophen (APAP) hepatotoxicity (300 mg/kg). The authors concluded that GM is hepatoprotective against acetaminophen-induced liver injury due to its antioxidant properties and anti-apoptotic capacity. A comparison of GM to the well-established clinically used antidote *N*-acetyl-*L*-cysteine (NAC) resulted in similar beneficial effects. According to the authors, their data suggest that GM is a potent hepatoprotective agent with a comparable mechanism to NAC. However, some of these mechanistic conclusions are clearly not justified by the data and whether or not GM is actually hepatoprotective under clinically relevant conditions has not been established.

The first concern is the authors' conclusion that GM has an anti-apoptotic effect. Apoptotic cell death is characterized by cell shrinkage and nuclear condensation[2,3]. However, none of these morphological features was evident from the liver sections shown by Wang *et al*[1]. In contrast, extensive centrilobular necrosis was shown, which correlates with the extensive release of liver enzymes into the plasma. The cells that are stained positive for DNA strand breaks with the transferase-mediated

dUTP nick end-labeling (TUNEL) assay are not apoptotic. These cells show the characteristic nuclear and cytosolic staining of oncotic cells, which has been extensively described for APAP hepatotoxicity[2-4]. The nuclear fragmentation is caused by the mitochondrial release of endonuclease G and apoptosis-inducing factor[4,5], rather than by caspase-activated DNase, which is responsible for DNA fragmentation during apoptosis[6]. Although the authors claimed that caspase-3 was activated based on the appearance of a 17 kD cleavage product, no other bands (pro-caspase-3) have been shown and no positive controls as reference points were included. Most importantly, a dramatic increase in procaspase-3 cleavage should go along with a dramatic increase in caspase-3 enzyme activity, which has never been shown in APAP-induced liver injury[2,7-9]. Consistent with these observations, caspase inhibitors do not protect against APAP hepatotoxicity arguing against a relevant role of caspase activation in the pathophysiology[8,9]. Furthermore, in both primary cultured mouse[10] and human hepatocytes[11], APAP induces necrotic cell death. Taken together, oncotic necrosis is the predominant mode of cell death (> 95%) during APAP hepatotoxicity in animal models *in vivo* and in humans[2]. The only exception is APAP-induced cell death in metabolically incompetent hepatoma cell lines. However, these mechanisms have no relevance to the *in vivo* hepatotoxicity of this drug[7].

A second major concern is related to the extensive mechanistic conclusion including the hypothesis that GM acts as an antioxidant. There is direct evidence that reactive oxygen species and peroxynitrite are formed in mitochondria during APAP hepatotoxicity and play a critical role in cell death[4,12,13]. However, the fact that at 12 h after APAP administration, there was less lipid peroxidation, together with other evidence for reduced tissue injury, does not prove that GM acts as an antioxidant. The same results would be obtained if GM protected and improved cell viability through other mechanisms with the consequence of less oxidant stress. In fact, one of the most likely mechanisms of protection, i.e. that one or several compounds in this plant extract may have inhibited cytochrome P450 activities or may have competed with APAP for metabolism, was not investigated. Toxicity of APAP is entirely dependent on its metabolic activation[14], which means that any interference with its reactive metabolite formation will substantially reduce or even eliminate toxicity. In the absence of clear evidence that this extract does not affect reactive metabolite formation, any conclusion regarding more distal mechanisms is not justified.

The third concern is the conclusion that GM acts as a hepatoprotectant similar to NAC. However, in clinically relevant situations of drug overdose, the antidote has to be effective when administered after the insult not as a pretreatment. The effectiveness of GM against APAP hepatotoxicity when treated after drug overdose has not been investigated. Furthermore, the comparison to NAC is not justified. NAC given as pretreatment to fasted animals will support glutathione (GSH) synthesis in the liver resulting in much higher GSH levels than the respective controls 2 h later[15]. These elevated GSH levels will more effectively scavenge the reactive metabolite of APAP and therefore prevent initiation of liver injury[16]. This protection mechanism of NAC is independent of the antioxidant effect of GSH mainly because no oxidant stress is generated at this point. However, if NAC is administered several hours after APAP, i.e. at a time when hepatic GSH is depleted and mitochondria have already generated an oxidant stress, GSH synthesized at this time is used to scavenge reactive oxygen species and peroxynitrite[13,17]. In addition, some of the excess NAC will also be used to support the impaired mitochondrial energy metabolism[17]. Both mechanisms contribute to the late protection against APAP hepatotoxicity[13,17]. Thus, NAC can have 3 different mechanisms of action depending on the time of administration relative to APAP. The pretreatment with NAC as used by Wang *et al*[1] will mainly scavenge the reactive metabolite of APAP, an effect that is unlikely to be relevant to GM.

Taken together, the protective effect of GM against APAP hepatotoxicity is an interesting observation. However, GM as a hepatoprotectant against drug toxicity under clinically relevant conditions has not been demonstrated. In addition, the actual protection mechanism of GM remains unclear. More mechanistic studies considering clinically relevant conditions are needed to evaluate the potential of this plant extract as an antidote against drug hepatotoxicity.

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Meetings

Events Calendar 2010

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Tamilnadu, India
International Conference on Medical Negligence and Litigation in Medical Practice

January 25-29
Waikoloa, HI, United States
Selected Topics in Internal Medicine

January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology Conference

January 28-30
Hong Kong, China
The 1st International Congress on Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of Gastroenterology & Endoscopy Meeting

March 09-12
Brussels, Belgium
30th International Symposium on Intensive Care and Emergency Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian National Association for Study of the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on Diabetes PACD14

March 25-28
Beijing, China
The 20th Conference of the Asian Pacific Association for the Study of the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology and Hepatology Conference, EGHC 2010

April 14-17
Landover, Maryland, United States
12th World Congress of Endoscopic Surgery

April 14-18
Vienna, Austria
The International Liver Congress™ 2010

April 28-May 01
Dubrovnik, Croatia
3rd Central European Congress of surgery and the 5th Croatian Congress of Surgery

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual Meeting

May 06-08
Munich, Germany
The Power of Programming: International Conference on Developmental Origins of Health and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and Rectal Surgeons Annual Meeting

June 04-06
Chicago, IL, United States
American Society of Clinical Oncologists Annual Meeting

June 09-12
Singapore, Singapore
13th International Conference on Emergency Medicine

June 14
Kosice, Slovakia
Gastro-intestinal Models in the Research of Probiotics and Prebiotics-Scientific Symposium

June 16-19
Hong Kong, China
ILTS: International Liver Transplantation Society ILTS Annual International Congress

June 20-23
Mannheim, Germany
16th World Congress for Bronchoesophagology-WCBE

June 25-29
Orlando, FL, United States
70th ADA Diabetes Scientific Sessions

August 28-31
Boston, Massachusetts, United States
10th OESO World Congress on Diseases of the Oesophagus 2010

September 10-12
Montreal, Canada
International Liver Association's Fourth Annual Conference

September 11-12
La Jolla, CA, United States
New Advances in Inflammatory Bowel Disease

September 12-15
Boston, MA, United States
ICAAC: Interscience Conference on Antimicrobial Agents and Chemotherapy Annual Meeting

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Prague, Czech Republic
Prague Hepatology Meeting 2010

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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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4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/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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EDITORIAL

Sedation and analgesia in gastrointestinal endoscopy: What's new?

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Abstract

Various types of sedation and analgesia technique have been used during gastrointestinal endoscopy procedures. The best methods for analgesia and sedation during gastrointestinal endoscopy are still debated. Providing an adequate regimen of sedation/analgesia might be considered an art, influencing several aspects of endoscopic procedures: the quality of the examination, the patient's cooperation and the patient's and physician's satisfaction with the sedation. The properties of a model sedative agent for endoscopy would include rapid onset and offset of action, analgesic and anxiolytic effects, ease of titration to desired level of sedation, rapid recovery and an excellent safety profile. Therefore there is an impulse for development of new approaches to endoscopic sedation. This article provides an update on the methods of sedation today available and future directions in endoscopic sedation.

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Key words: Gastrointestinal endoscopy; Analgesia; Sedation; Propofol

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INTRODUCTION

The best methods for analgesia and sedation during gastrointestinal endoscopy are still debated. Providing an adequate regimen of sedation/analgesia might be considered an art, influencing several aspects of endoscopic procedures: the quality of the examination, the patient's cooperation and the patient's and physician's satisfaction with the sedation[1].

Ensuring adequate sedation and analgesia and the patient's clinical stability by appropriate monitoring during endoscopic procedures has become extremely important over the last 10 years or so[2,3]. Open-access endoscopy, where no outpatient evaluation is required before the procedure, is increasingly common. Patients, sometimes with comorbidities, arrive for screening or routine endoscopy without having been assessed for pathologies that might influence procedural sedation. In addition, in situations where the examination is more invasive or time-consuming, the level of sedation must be optimized in order to achieve ideal procedural conditions.

The best sedation strategy should be tailored to the individual patient, based on the clinical risk evaluation and the type of procedure to be done[3-5]. Sedation can be defined as a drug-induced depression in the level of consciousness. Sedation and analgesia comprise a continuum

of states ranging from minimal sedation (anxiolysis) through general anesthesia. The majority of endoscopic procedures are done under a regimen of moderate sedation and the literature suggests that combining a benzodiazepine with an opioid provides an effective level of sedation. Targeting a moderate level of sedation gives a better overall profile than a deeper level and should also give a better safety margin for a non-anesthesiologist[3]. However, Patel *et al*[6], measuring sedation levels during endoscopic procedures performed with a combination of meperidine and midazolam, observed that unintended excursion into a deeper level of sedation did not generally result in adverse outcomes and suggest the safety margin is wide around targeted moderate sedation.

An important general principle about achieving moderate sedation with a combination of drugs is that the combined effect of an opiate and benzodiazepine is much greater than the additive effects of the single drugs so there is a greater risk of complications[7].

The target level of sedation and the agents chosen will depend on the characteristics of the endoscopic procedure (length and painfulness), individual patient's factors (age, comorbidities, anxiety, *etc.*), patient's preferences, and the need for cooperation[8]. A critical point for the non-anesthesiologist is that although a patient's characteristics help establish the target dosage, it is impossible to predict accurately the exact dose needed to complete the procedure. Even when the blood levels of a particular drug are similar, one patient's experience of sedation can be quite different from that of another. Therefore, a successful outcome is dependent on an understanding of incremental dosing, the synergistic effects of drug classes, and the onset of action and peak effects of sedation agents. In addition, clinicians must always be prepared to rescue patients who move to the next deeper level of sedation[7].

Propofol is a short-acting intravenous agent with a rapid onset of action, short recovery profile, anti-emetic proprieties and good amnesic effects. Several studies have suggested that propofol offers significant advantages over benzodiazepines and opioids for sedation during endoscopic procedures and two large prospective studies indicated it was more effective and safer than midazolam and meperidine for reaching and maintaining an adequate level of sedation during endoscopic procedures, resulting in better titration of the level of sedation, and shorter recovery times[9-14].

In 2004 the American College of Gastroenterology, American Gastroenterology Association and the American Society for Gastrointestinal Endoscopy issued a joint statement affirming that large case studies indicate that adequately trained nurses, supervised by a physician, can safely and effectively administer propofol. Worldwide experience with gastroenterologist-directed sedation with propofol combined with improvements in our understanding of its dosing and titration for moderate sedation, as stated by Cohen *et al*[15], have prompted several medical societies to question the real necessity of restricting its use to anesthesiologists. In 2004 the American Society for Gastrointestinal Endoscopy, in its Training Guidelines for Use of Propofol in Gastrointestinal Endoscopy, stated: "While properly trained physicians can administer propofol, regulations governing its administration by non-physician personnel are variable on a state-by-state basis"[4,15]. Nevertheless, in April 2004 the American Society of Anesthesiologists (ASA) recommended that, when propofol is used for moderate sedation and analgesia, it should be administered by someone trained in administering general anesthesia. This is in line with the "black box" warning for propofol, in which the manufacturer makes the same recommendations. Furthermore, in 2005 the ASA stated that: "Propofol is an anesthetic drug and the ASA believes that the involvement of an anesthesiologist in the care of every patient undergoing anesthesia is optimal"[16].

In a recent update article Rex *et al*[17] opined that given the big financial incentives from special interest groups, such as the anesthesiologists' community, it is unlikely that non-anesthesiologist-directed propofol administration will expand dramatically in the near future.

NON-ANESTHESIOLOGIST PROPOFOL SEDATION

Many studies address the safe and effective administration of propofol during gastrointestinal endoscopy, by either physicians or trained nurses. In 2005 the cumulative reported experience with nonanesthesiologists-administered propofol during gastrointestinal endoscopic procedures was more than 80 000 patients[18-26]. In these reports there were no patients requiring endotracheal intubation or resulting in death. In 2005, in a large prospective trial involving three endoscopy units (Indiana, Oregon and Switzerland) and 36 743 cases of nurse-administered propofol sedation, the authors concluded: "Trained nurses and endoscopists can administer propofol safely for endoscopic procedures. Nurse-administered propofol sedation is one potential solution to the high cost associated with anesthesist-delivered sedation for endoscopy" and moreover: "It remains uncertain whether large prospective series of NAPS with safe performance will satisfy the concerns of anesthesia societies regarding the safety of propofol administration for endoscopy by appropriately trained registered nurse/endoscopy teams"[25].

In 2007 Cohen and the AGA Institute, reviewed the standard practice of endoscopic sedation and, regarding the use of gastroenterologist-directed propofol, recommended that: (1) Gastroenterologist-directed propofol sedation is medicolegally reasonable, but requires appropriate endoscopist training, patient selection and adherence to protocols for administration as well as compliance with institutional and local regulation; and (2) The endoscopist should be ACLS certified and provide sedation in keeping with expert practice guidelines and with institutional and state guidelines. Endoscopy Units should conform to practice guidelines regarding procedure-related sedation, including documentation, training of staff, maintenance of rescue equipment, creation of appropriate emergency protocols and quality assurance programme[15].

In 2008 Rex, in an update about gastroenterologist-directed propofol, summarized published literature on the safety of gastroenterologist-directed propofol reporting more than 220 000 cases without a single reported death. In only 1 case endotracheal intubation was required during endoscopic retrograde cholangiopancreatography (ERCP) with full patient's recovery. As stated by Rex, despite evidence that there are large case series supporting the use of propofol sedation by non-anesthesiologists, numerous obstacles have persisted regarding its expansion; most prominent of these is the institutional control that anesthesiologists maintain over sedation policies[17]. The ASA Task Force recommends that patients receiving propofol should receive care consistent with deep sedation and that those personnel should be capable of rescuing the patient from general anesthesia[2]. However, there is abundant evidence that propofol can be administered safely by non-anesthesiologist.

In a recent paper by Rex *et al*[26], a world-wide multicenter safety review of 646 080 (223 656 published and 422 424 unpublished) endoscopist-directed propofol sedation cases was conducted. Endotracheal intubation, permanent neurologic injuries and deaths were 11, 0 and 4, respectively. The 4 deaths occurred in patients with significant comorbid illness. In this series endoscopist-directed administration of propofol appears to have a lower mortality rate than that in published data on traditional sedation with benzodiazepines and opioids and a comparable rate to that on general anesthesia by anesthesiologists.

The Endoscopic Section of the German Society for Digestive and metabolic Diseases have recently published the Guideline for Sedation for Gastrointestinal Endoscopy 2008. This guideline states: "For simple endoscopic examinations and in low-risk patients, sedation (with propofol) should be induced by a properly qualified physician and can then be monitored by an experienced person with appropriate training. The person must not have any other tasks while monitoring the sedation. Propofol may be administered by a properly trained and experienced person who has this as his or her sole task (recommendation grade A, strong consensus)"[28]. Furthermore, the literature findings on the safety of endoscopist-administered show that the use of an anesthesiologist for sedation for endoscopic procedures is costly. The recommendation of the German guideline is still that calling in an anesthesiologist should be considered only for patients with a high risk profile such as high ASA grade (Ⅲ-Ⅳ), or pathological and anatomical features associated with a higher risk of airway obstruction during the intervention.

Recently, the American Association for the Study of Liver Disease, American College of Gastroenterology, American Gastroenterological Association and American Society for Gastrointestinal Endoscopy have released the "Position statement: nonanesthesiologists administration of propofol for GI endoscopy" published in 2009 December, which concludes that the administration by non-anesthesiologists of propofol *vs* standard sedation with benzodiazepines and opioids is comparable with respect to their efficacy and safety profile[29].

Proper training and patient selection are mandatory for the safe practice of nonanesthesiologists-administered propofol sedation.

The Sedation Task Force, which was chaired by Cohen LB and included representatives from each of the four societies, was convened to develop a document designed to provide an evidence-based assessment of propofol-mediated sedation by properly trained nonanesthesiologists. The document was approved by the governing boards of all four societies.

A number of recommendations are made in the statement regarding non anesthesiologist propofol sedation (NAPS): (1) The safety profile of NAPS is equivalent to that of "standard sedation" with respect to the risks of hypoxemia, hypotension and bradycardia for upper and lower endoscopy, ERCP and endoscopic ultrasound (EUS). The worldwide experience with NAPS during EUS and ERCP, however, is insufficient to draw definitive conclusions about its use in these setting; (2) For upper and lower endoscopy, ERCP and EUS, the time for sedation induction and the recovery time using NAPS are shorter in comparison to that with standard sedation; patient satisfaction is equivalent or slightly superior; and (3) For ERCP and EUS, NAPS is more cost-effective than standard sedation. The use of anesthesiologist-administered sedation for healthy, low-risk patients undergoing routine gastrointestinal endoscopy results in higher costs with no proven benefit with respect to patient safety or procedural efficacy.

NAPS requires the acquisition of skills and abilities that are distinct from those necessary for standard sedation. Training programs that are both didactic and practical should be provided by Scientific Societies.

Although current methods of sedation are effective for the majority of patients undergoing endoscopy, techniques that enhance patient satisfaction, safety and recovery are desirable.

Infusion platforms that permit control of drug delivery by the patient are promising alternatives to current methods of sedation.

PROPOFOL INFUSION PLATFORMS

New concepts in endoscopic sedation include enhanced mechanisms for drug delivery such as target-controlled infusion (TCI) and patient-controlled sedation or analgesia (PCS or PCA). The TCI pump, first described in the 1980s, provides infusions based on pharmacokinetic models of the specific drug, using a computer-controlled pump; the target concentration (in μg/mL) is automatically achieved and maintained over time by varying the infusion rate according to a three-compartment pharmacokinetic model, with very good predictive performance. The infusion rate is directly and automatically adapted by the software managing the pump, without the need for any of the complex calculations required for a manual scheme.

The ASA guidelines recommend accurate titration of sedative/analgesic medications to improve patients' comfort and safety and avoid the risk of over-sedation[2]. Among different systems available for administration of

propofol, the TCI pump is undoubtedly one of the most sophisticated and several studies have shown that target-controlled drug infusion ensures excellent and safe sedation during endoscopic procedures[30-32].

For PCS or PCA, the patient self-administers the medication in response to pain; therefore, the patient must be conscious enough to press the hand-held button. A lock-out time is programmed in order to prevent the delivery of additional doses until the previous dose has taken full effect. PCA, which allows the patient to self-administer frequent, small doses of opioids, as needed to manage pain, could be an effective method for pain control during gastrointestinal endoscopy[33-35].

Pambianco *et al*[36] reported on the investigational computer-assisted personalized sedation system with propofol (CAPS), designed to enable physician-nurse teams to keep patients undergoing endoscopic procedures at minimal to moderate sedation levels, preventing them slipping into deep sedation with drops in cardio-respiratory function. The report described two open-label single-center trials of the CAPS device on 48 patients undergoing either colonoscopy or EGD procedures and found that the results were reproducible across two countries and two practice settings. The system continuously monitors six parameters including oxygen saturation, respiratory rate, heart rate, non-invasive blood pressure, end-tidal carbon dioxide and patients' responsiveness to verbal and tactile stimuli. In this trial the moment-to-moment control of propofol infusion led to very rapid post-procedure recovery from sedation (< 30 s), and the system, also known as SEDASYS™, performed as designed, reducing or stopping propofol infusion at the first signs of over-sedation, with a very low incidence of desaturation. Pambianco *et al*[36] said that the CAPS device detected apnea more sensitively than clinical observation. In their trial there was no need for airways management or mechanical ventilation.

The CAPS device facilitates the titration of propofol to the desired clinical effect by automatically calculating and delivering a loading dose. The platform continuously checks for early signs of potential adverse effects and the level of sedation, so the physician/nurse teams can adjust the infusion as required, to the targeted degree of sedation. The system is also designed to respond to early signs of over-sedation, as indicated by apnea or hypoxemia, by stopping or reducing delivery of propofol, increasing oxygen delivery and automatically instructing patients to take a deep breath.

In two feasibility studies of CAPS involving 96 patients undergoing elective upper endoscopy and colonoscopy, patients remained minimally to moderately sedated throughout the procedure. For the majority, recovery time was less than 1 min and there were no serious adverse events.

The nurses assisting in the procedures reported that the system was intuitive and user-friendly, and the built-in dosing limits and automated response algorithms allowed the team to be confident that an appropriate level of sedation was being maintained.

A multi-center prospective, randomized, controlled pivotal trial, presented at Digestive Disease Week in May 2008, compared the safety and effectiveness of propofol plus a single dose of fentanyl administered with the CAPS system, or physician-administered standard sedation regimens involving opioids and benzodiazepines (midazolam with either fentanyl or meperidine) in 1000 patients. Patients who received CAPS sedation had significantly lower measures of cumulative oxygen desaturation than those assigned standard sedation). There were 34 adverse events, no serious adverse events and no rescue interventions among patients sedated with CAPS; there was one rescue intervention in the control group[37]. As stated by Pambianco *et al*[37], the trial investigators, the system made it possible to maintain minimal to moderate sedation with propofol during upper endoscopy and colonoscopy, and helped prevent patients entering deep sedation, which is traditionally associated with propofol.

The system seems to offer a way to personalize the level of sedation appropriate for each patient because it combines propofol delivery with sophisticated monitoring for better control of the sedation regimen, by predicting the patient's level of sedation. Based on the findings of these investigations, the system has been submitted to the US FDA for pre-marketing approval under the brand name SEDASYS™ System.

Most states require the presence of an anesthesiologist during propofol administration, an expensive and unfavorable arrangement for most gastrointestinal endoscopy centers. Current propofol black-box labeling states that only persons trained in general anesthesia should administer the drug. However, pending a favorable FDA review, labeling for the CAPS system will allow physicians and nurses to administer propofol sedation for endoscopic procedures, without the assistance of an anesthesiologist.

In the light of the increasing use of propofol by non-anesthesiologists, some anesthesiologists remain sceptical about CAPS systems, asserting that they cannot substitute for a trained professional, particularly in an emergency. In view of the specific risks of propofol, even if only moderate sedation is intended, patients receiving the drug should receive care consistent with that required for deep sedation, considering the potential for abrupt onset of airway obstruction and apnea and the lack of a specific antidote.

As stated by Iravani[38] in a recent editorial, "the intriguing question is whether the use of CAPS in administering propofol prevents the progression of sedation to unintended depths, deep sedation or general anesthesia. In other words, does this precise moment-to-moment control of the propofol infusion titrated to clinical effect obviate the need for the presence of personnel proficient in airway management and advanced life support?".

Could this technology facilitate access to propofol sedation by gastroenterologists? The Anesthesia and Respiratory Device Review panel met on May 28, 2009 and, by an 8-2 margin, approved the CAPS platform for use by endoscopist/nurse teams in EGDS/colonoscopy in ASA Ⅰ / Ⅱ adults under the age of 70 years, with BMI < 35 kg/m^2.

Major hurdles that still remain for propofol adoption are the diversity of medical society guidelines, the costs of anesthesiologist-attended endoscopic procedures, and the immutable, indelible black-box warning about propofol administration. Furthermore, until there is a training program for the use of propofol by non-anesthesiologists, FDA approval of CAPS will probably not be forthcoming.

DRUGS: WHAT'S NEW?

A number of prodrug formulations of propofol have been developed to overcome the disadvantages of the current lipid-based formulations, including stability, the potential complications of lipid infusion, the risk of contamination and fluctuations in propofol plasma levels due to the bolus injection.

Fospropofol disodium (FD), which now goes by the brand names of Lusedra or Aquavan, is a water-soluble prodrug of propofol with pharmacokinetic and pharmacodynamic properties that differ from propofol emulsion. FD is the first propofol prodrug to be studied for moderate sedation. After IV injection, propofol is released from FD by tissue alkaline phosphatases with a predictable pattern of plasma concentrations, resulting in lower peak concentrations and a more gradual decline in drug concentrations than with a standard propofol injection. Due to the different and complex pharmacokinetics of FD, time to peak sedative effect after a bolus injection is between 3 and 7.5 min, compared with 1 min 36 s for propofol[39].

Although the anesthesiologist community has shown only limited interest, FD has been investigated for sedation by non-anesthesiologists. In 2008 Cohen stated in a review that "FD may be an attractive agent for gastroenterologists interested in realizing the benefits of propofol without the requirement of an anesthesia specialist in the endoscopy suite"[40]. In July 2008 the FDA approved FD with a "MAC label", which means that this drug, like propofol, will be used mostly by anesthesiologists and certified registered anesthetist nurses. The drug manufacturer has been in discussions with FDA regarding additional studies and remains committed to getting the product approved for use by non-anesthesiologists.

The ideal drug for sedation for non-anesthesiologists should have certain properties to ensure safe, effective sedation. These include a predictable pharmacokinetic profile, rapid onset of action, analgesic and anxiolytic effects, short recovery time, minimal associated risks and no requirement for the presence of an anesthesiologist. Ketamine[41,42], nitrous oxide[43,44] and dexmedetomidine[45,46] have all been studied for procedural sedation. While some results have been promising, none fulfil all these criteria or offer any true advance in sedation during gastrointestinal endoscopy.

Remifentanil, an ultra-short-acting μ-opioid receptor agonist, has some advantages over other opioids because of its rapid onset and offset times, making it suitable for controlling pain during endoscopic procedures[35,47,48]. The combination of rapid onset and offset of action translates clinically to easy titrability. However, its respiratory depressant effect has been amply reported[49,50]. On account of this, remifentanil is typically administered in a moderate sedation/analgesia setting, by continuous infusion using a programmable infusion pump. There are many reports of the use of remifentanil in different settings (obstetrics, gastrointestinal endoscopy, pain control after abdominal surgery) using PCA, with or without background infusion, and the quality of analgesia and patient satisfaction seem to be the same as with standard sedation/analgesia[35,52-55].

New sedatives and delivery systems under development have the potential to improve the quality of endoscopic sedation/analgesia. Scientific societies now need to establish training schemes for the use of sedatives and delivery systems. These should include training in advanced cardiac life support, the pharmacokinetics and pharmacodynamics of the drugs to be used, airway assessment, and training in simple measures of ventilatory support in patients with apnea or airway impairment during sedation for gastrointestinal endoscopy[15].

CONCLUSION

Most guidelines all over the world recommend the sedation of patients when undergoing gastrointestinal endoscopy. Benzodiazepines combined with opioids are the most frequently used sedative agents; however there are a number of potential concerns/problems with available sedative agents. In fact, most complications in gastrointestinal endoscopy are not related to the procedure, but to sedation and include cardiopulmonary events such as hypoxemia, hypoventilation, airway obstruction, apnea, arrhythmias, hypotension and vasovagal episodes. Finding an ideal regimen of sedation seems to be similar to "Searching the Holy Grail". Since the introduction of propofol in the 1980s, for the induction and maintenance of anesthesia, its clinical application have expanded to include monitored anesthesia care and procedural sedation. The use of propofol for endoscopic sedation has increased markedly during the past 10 years. Several studies have established that propofol is superior to traditional sedative regimens because of its superior recovery profile and its safety profile.

The worldwide safety experience of endoscopist-administered propofol sedation now exceeds 460 000 patients. We look forward to a time when a sedation team will be qualified to administer propofol during gastrointestinal endoscopy without the presence of the anesthesiologist, following a proper training program. The new emerging technology could facilitate access to propofol sedation.

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EDITORIAL

Pyogenic liver abscess: Differences in etiology and treatment in Southeast Asia and Central Europe

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Abstract

Knowledge of etiology and timely treatment of underlying causes, when possible, play an important role in the successful therapy of patients with pyogenic liver abscess (PLA). Recent publications from Central Europe and Southeast Asia hint at considerable differences in etiology. In this article, we aim to elaborate these differences and their therapeutic implications. Apart from some special types of PLA that are comparable in Southeast Asia and Central Europe (such as posttraumatic or postprocedural PLA), there are clear differences in the microbiological spectrum, which implies different risk factors and disease courses. *Klebsiella pneumoniae* (*K. pneumoniae*) PLA is predominantly seen in Southeast Asia, whereas, in Central Europe, PLA is typically caused by *Escherichia coli*, *Streptococcus* or *Staphylococcus*, and these patients are more likely to be older and to have a biliary abnormality or malignancy. *K. pneumoniae* patients are more likely to have diabetes mellitus. Control of septic spread is crucial in *K. pneumoniae* patients, whereas treatment of the underlying diseases is decisive in many Central European PLA patients.

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Key words: Liver abscess; *Klebsiella pneumoniae*; Biliary tract diseases; Drainage; Antibiotics

INTRODUCTION

Pyogenic liver abscess (PLA) is the result of bacterial infection of the liver parenchyma, with subsequent infiltration by inflammatory cells and formation of a collection of pus. It is still a severe disease with considerable mortality (6%-14%)[1,2]. Chen *et al*[3] recently have reported an intensive care unit mortality rate of 28%. Treatment policies have changed in recent decades as a result of developments in interventional radiology that offer imaging-guided percutaneous puncture and drainage as procedures that are less invasive than surgical therapy. However, several studies have shown that underlying diseases and the overall condition of the patients have major prognostic significance[4,5]. Both the APACHE (acute physiology and chronic health evaluation) II and the SAPS (simplified acute physiology score) II scoring methods are appropriate for assessing mortality of PLA patients[6]. Thus, knowledge of etiology and timely treatment of underlying causes, when possible, play an important role in successful treatment. Recent publications from Central Europe and Southeast Asia hint at considerable differences in etiology. This editorial aims to elaborate these differences and their therapeutic implications. Information from recent publications on liver abscesses from PubMed was used. *Klebsiella* PLA is mainly described in series from Southeast Asia, whereas

European and American studies usually comprise a mixture of different etiologies. Special interest was placed on publications devoted to characteristics and specific features of *Klebsiella* abscesses as compared to those of other origin.

GENERAL CONSIDERATIONS ON CLINICAL MANAGEMENT

Antibiotic treatment is the basis of clinical PLA management. Gram stain, abscess culture (with sensitivity testing) and blood culture provide valuable information to guide successful therapy. Chemaly *et al*[7] have found that the sensitivity and specificity of Gram stains of liver abscess material were 90% and 100% for Gram-positive cocci (GPC) and 52% and 94% for Gram-negative bacilli (GNB). The sensitivities of the blood cultures for any GPC and GNB present in the liver abscess were 30% and 39%, respectively. Of the positive blood cultures, 35% contained organisms not recovered from abscess cultures. On the other hand, PLA pathogens are usually underrepresented in blood cultures. In a recent study from Pakistan with a total of 966 patients, 540 responded to medical therapy, whereas adjunctive percutaneous aspiration was performed in 426. Predictive factors for aspiration of liver abscess were: age $\geqslant$ 55 years, size of abscess $\geqslant$ 5 cm, involvement of both lobes of the liver, and duration of symptoms $\geqslant$ 7 d[8]. Microbiology also hints at pathogenesis: local spread typically leads to mixed flora[9], and hematogenous seeding to a single microorganism (typically *Staphylococcus aureus* or *Streptococcus* spp.).

Apart from choosing the right antibiotic, another crucial concern is whether a sufficient concentration of the antibiotic reaches the target site. In this context, Matoba *et al*[10] have published an article on intermittent hepatic artery antibiotic infusion therapy, especially for patients in whom percutaneous drainage could not be performed and intravenous administration of antibiotics was ineffective. The value of this treatment (for a highly selected patient group) still has to be shown with a larger number of patients.

As for abscess drainage, the percutaneous technique has often come to replace surgical procedures. Surgery nonetheless still plays an important role when percutaneous drainage fails[11] or when underlying pathology such as biliary disease and malignancy has to be addressed[12]. In selected cases, drainage may also be done laparoscopically[13] or *via* a trocar in local anesthesia[14]. Hsieh *et al*[15] have found that aggressive hepatic resection for patients with liver abscesses and APACHE Ⅱ scores of $\geqslant$ 15 produce better clinical outcomes than other therapeutic options.

PLA IN CENTRAL EUROPE

Our patients[5] show the etiological spectrum typical for our region, with biliary disorders first, followed by cryptogenic abscess, PLA due to systemic infection, and posttraumatic origin. Some other, rarer causes were also identified (portal route of infection, hydatid origin, postoperative/postprocedural, foreign body). The microorganisms found most frequently were *Staphylococcus*, *Streptococcus* and *Escherichia coli* (*E. coli*). *Klebsiella* spp. were only found in 5% of cases. In about 25% of the patients, the abscesses were sterile and this was at least partly due to antibiotic pretreatment. Anaerobic agents are encountered with increasing frequency but this might be attributable to improved transport and culture techniques. A recent study from Spain[16] has shown similar results, with *Streptococcus* and *E. coli* found most frequently, but a higher number of *Klebsiella* spp. (14%). The most common complication was pleural effusion.

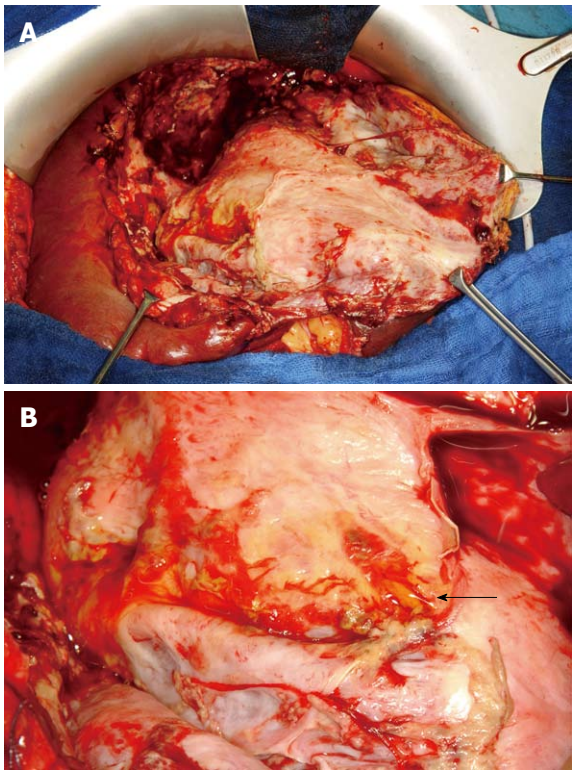


Figure 1 Posttraumatic liver abscess. A: Intraoperative image after evacuation of putrid fluid; B: Detail from the floor of the abscess cavity showing a biliary fistula (arrow).

Prolonged disease course may result from unexpected or particularly aggressive agents, underlying pathology or constant inflammatory stimuli provoked by a foreign body. As a rare cause of pyogenic abscesses, *Salmonella* infection (hematogenous or ascending *via* the biliary tree) has been described[17-23].

Underlying pathology is frequently found in our region and is often the crucial prognostic factor. This includes gallbladder empyema, biliary fistula[24] (Figures 1 and 2), pylephlebitis secondary to acute appendicitis[25,26], and malignancy (e.g. hepatocellular carcinoma, cholangiocarcinoma, liver metastases, gallbladder carcinoma, carcinoma of the papilla, and pancreatic cancer). Operative treatment of the underlying disease is indicated whenever feasible. For the healing of biliary fistula, good bile

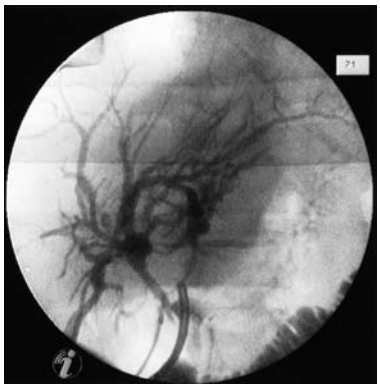


Figure 2 Intraoperative cholangiogram (injection of contrast medium into the fistula ostium).

flow is crucial and may be provided by papillotomy and biliary stenting. In patients with cryptogenic PLA, silent abdominal pathology should always be considered and further studies such as colonoscopy are recommended[27].

In special cases, a foreign body penetrating from the digestive tract may provide a constant stimulus for inflammation. Several kinds of foreign bodies such as toothpick[28,29], pin[30], needle, bone[31,32], pen, dental plate, clothespin, wire crucifix, and rosemary twig[33] have been described[9]. Removal of the foreign body is crucial for successful treatment.

PLA IN SOUTHEAST ASIA

Klebsiella pneumoniae (*K. pneumoniae*) liver abscess[34-42] recently has been reported as the leading cause of PLA in Korea and Taiwan, and is described as a globally emerging infectious disease. *K. pneumoniae* is found as a harmless commensal but is also a frequent nosocomial pathogen (causing urinary, respiratory and blood infections) and the agent of specific human infections including Friedländer's pneumonia, rhinoscleroma and PLA[43].

Literature on this entity predominantly comes from Southeast Asia but it has also been reported as an emerging problem in North America[44]. In a recent report from New York, 60% of the patients with *Klebsiella* PLA were of Asian origin, although many of them had not resided in their country of origin for quite some time[45]. Although *K. pneumoniae* is prevalent in patients with diabetes, it can also attack healthy people or alcoholics[46]. Foo *et al*[47] have found that diabetes mellitus is not a predictor of fatality. The MagA virulence gene has been described in invasive strains. According to Yeh *et al*[48], and in accordance with the bacterial polysaccharide gene nomenclature scheme, MagA should be renamed Wzy (KpK1), the capsular polymerase specific to *K. pneumoniae* serotype K1. Gas formation by mixed acid fermentation of glucose is a typical feature.

K. pneumoniae PLA constitutes a clinical entity distinct from polymicrobial liver abscess, has a high propensity to metastasize, and rarely is associated with intra-abdominal abnormalities. PLA-producing strains are commonly resistant to ampicillin. Complications include metastatic meningitis, endophthalmitis, acute respiratory distress syndrome, septic pulmonary emboli, and necrotizing fasciitis. Braiteh *et al*[49] have described three distinct *K. pneumoniae* liver abscess syndromes: the polymicrobial liver abscess, the monomicrobial cryptogenic noninvasive liver abscess, and the monomicrobial cryptogenic invasive *K. pneumoniae*-associated liver abscess (CIKPLA) syndromes, with distinct clinical, epidemiological and outcome features. CIKPLA syndrome typically affects diabetic patients, mainly in Southeast Asia, and is complicated by septic spread to other organs.

Chen *et al*[50] have found that, in contrast to patients with *K. pneumoniae*, patients with *E. coli* liver abscess were more likely to be older and female, have a biliary abnormality or malignancy, pleural effusion, polymicrobial infection with anaerobic or multi-drug-resistant organisms, a higher APACHE Ⅱ score, and to have been treated initially with ineffective antibiotics. *K. pneumoniae* patients were more likely to have diabetes mellitus and the cause of infection was often cryptogenic. A higher APACHE Ⅱ score at admission and higher frequency of coexisting malignancy may have contributed to the higher mortality in *E. coli* PLA, but the difference was not statistically significant. Tsai *et al*[51] also have reported lower death rates for *Klebsiella* PLA. As for older patients, Chen *et al*[52] have found that they have a fair outcome but require longer hospitalization. After successful treatment, the recurrence rate of *Klebsiella* liver abscesses is reported to be low[53,54]. Kim *et al*[55] have described the differences in CT findings of *Klebsiella* and non-*Klebsiella* liver abscesses. The former has a series of characteristic intra-abscess features: septal breakage, turquoise-like structure, hairball-like content, air-fluid level, and no enhanced rim.

Another entity well known in endemic areas of echinococcosis, but only occasionally found in Central Europe, is liver abscess of hydatid origin[56]. It is usually associated with few symptoms, because the pericystic structure acts as a nonspecific defense mechanism. Anthelminthic drugs are of limited value.

POSTPROCEDURAL AND POSTTRAUMATIC PLA

These special forms of PLA are seen in Central Europe and South East Asia. Postprocedural PLA may occur after hepatic resection; formation of pus in the operation cavity is usually amenable to percutaneous drainage. Post-transplantation liver abscesses, however, pose more complex problems. The most common predisposing factor is hepatic artery thrombosis, which may lead to fulminant hepatic necrosis, delayed bile leakage or relapsing bacteremia. In contrast to the normal liver, the graft lacks natural attachments and collaterals. Mortality of this type of PLA is still high (42%)[57] and mean duration of drainage is long. PLAs may also occur after transarterial chemoembolization[58,59] for malignant hepatic tumors, particularly after biliary reconstructive surgery (aggressive antibiotic prophylaxis is recommended for these patients). They are also seen as rare complications after radiofrequency ablation[60-62] or cryosurgery[63].

About 5% of PLAs are of posttraumatic origin; they occur in 0.7%-1.5% of patients undergoing nonoperative treatment of liver trauma caused by bacterial seeding of hematoma or necrosis[64,65]. Risk factors include enteric injuries, persistent hemorrhage with excessive need of blood transfusions, and extensive parenchymal damage. The necessity for antibiotic prophylaxis in liver trauma patients remains an unresolved question; it might be justified with liver injury of at least grade 3 and is usually given with associated open wounds.

CONCLUSION

Apart from some special types of PLA that are comparable in Southeast Asia and Central Europe (such as posttraumatic or postprocedural PLA), there are clear differences in the microbiological spectrum, which also implies different risk factors and courses. *K. pneumoniae* PLA is predominantly seen in Southeast Asia, whereas, in Central Europe, PLA is typically caused by *E. coli*, *Streptococcus* or *Staphylococcus* and these patients are more likely to be older and to have a biliary abnormality or malignancy, pleural effusions, and a higher APACHE Ⅱ score. *K. pneumoniae* patients are more likely to have diabetes mellitus and the cause of infection is often cryptogenic. The typical complication for *Klebsiella* infection is metastatic spread to various organs. On the other hand, high APACHE Ⅱ scores at admission and a high frequency of coexisting malignancy may often contribute to a critical course in Central European PLA patients. Control of septic spread is crucial in *K. pneumoniae* patients, whereas treatment of the underlying diseases is a decisive issue in many Central European PLA patients.

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Clinical features of hepatitis B virus-related hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is a major cause of cancer death, and chronic hepatitis B is a serious worldwide problem. The epidemiology of HCC is distinctive. Hepatitis B virus (HBV) plays a major role in hepatocarcinogenesis. Prevention of HBV-related HCC is a key issue in current hepatology. This paper describes the prevention and clinical features of HBV-related HCC, along with a short review of the disease.

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Key words: Hepatocellular carcinoma; Hepatitis B virus

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INTRODUCTION

Persistent infection by hepatitis C virus (HCV) or hepatitis B virus (HBV) is a background factor in almost all cases of hepatocellular carcinoma (HCC) in Japanese patients, as it causes chronic hepatitis and liver cirrhosis, and ultimately HCC. Several studies have indicated that HCC develops mainly in the cirrhotic liver and alongside chronic liver diseases. It is widely accepted that viral cirrhosis caused by HBV or HCV infection is an important risk factor for the development of HCC. However, HCC can develop in non-cirrhotic livers, particularly in countries where HBV is endemic. The present paper discusses the clinical features of HBV-related HCC and the importance of viral status and viral eradication for improving the prognosis of HBV-related HCC.

CARCINOGENIC FACTORS AFFECTING CHRONIC LIVER DISEASE (TYPE B)

It is believed that the incidence of carcinogenesis is low in cases with fibrotic severity index F0 according to the new Inuyama classification of chronic hepatitis related to HBV, whereas it increases with a decrease in cure rate in association with an increase in the fibrotic severity index F. It is thus believed that the incidence of carcinogenesis also increases with increased severity of cirrhosis in patients with chronic hepatitis.

Accordingly, we examined carcinogenesis-modulating factors by long-term observation of patients with HBV-related liver cirrhosis[1].

Several studies have indicated that HCC develops mainly in the cirrhotic liver. It is widely accepted that viral cirrhosis due to infection by HBV or HCV is an important risk factor for the development of HCC. It has also been indicated that various factors and their interactions are involved in the etiology and pathogenesis of HCC. We thus determined the rate of HCC development to assess the risk factors for hepatocellular carcinogenesis in patients with HBV-related compensated viral cirrhosis.

Table 1 Carcinogenic factors involved in hepatocellular carcinoma in patients with type B cirrhosis[1]

Variable	*n*	Univariate analysis			Multivariate analysis		
		P-value[1]	RR[2]	95% CI	*P*-value[3]	RR[2]	95% CI
ALT (IU/L)							
⩾ 100	14	0.0083	2.044	1.098-4.677	0.025	4.525	1.202-17.030
< 100	51		1.000			1.000	
LDH (IU/L)							
⩾ 480	30	0.0138	2.000	0.878-4.558	NS (0.081)	2.880	0.881-9.412
< 480	35		1.000			1.000	
HBV-DNA (LGE/mL)							
⩾ 3.7	46	0.0082	2.836	1.269-6.340	0.014	7.712	1.511-39.365
< 3.7	19		1.000			1.000	

[1]*P*-values were obtained by using the log-rank test; [2]Relative risks were calculated by comparing classes using a Cox regression analysis; [3]*P*-value were obtained by using a Cox regression analysis. HBV: Hepatitis B virus; ALT: Alanine aminotransferase; LDH: Lactate dehydrogenase; NS: Not significant.

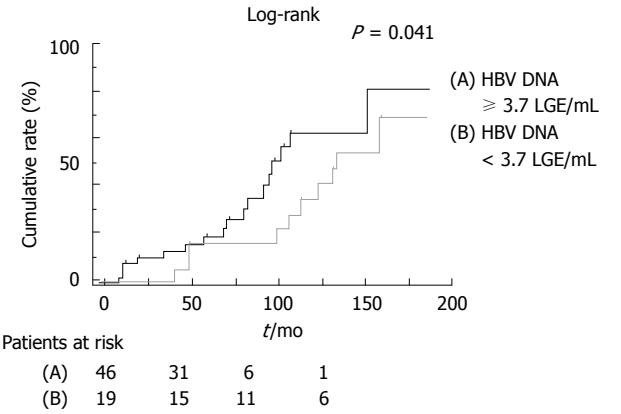


Figure 1 Comparison of the cumulative incidence of hepatocellular carcinoma by viral level in patients with type B cirrhosis[1]. HBV: Hepatitis B virus.

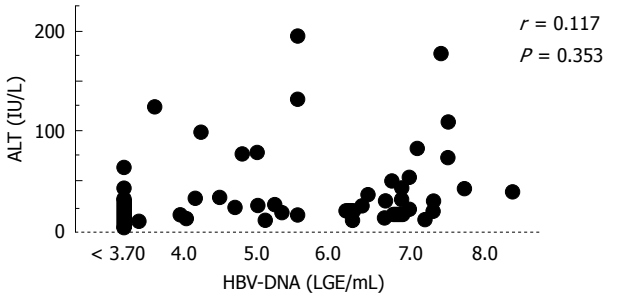


Figure 2 Correlation between HBV-DNA and alanine aminotransferase (ALT) levels in patients with type B cirrhosis[1].

We previously concluded that a high HBV-DNA titer (more than 3.7 LGE/mL) was the most predictive factor of the development of HCC in HBsAg-positive liver cirrhosis. It was found by univariate analysis that the incidence of carcinogenesis was affected by the following three factors with statistical significance: alanine aminotransferase (ALT) ⩾ 100 IU/L ($P = 0.008$), lactate dehydrogenase (LDH) ⩾ 480 IU/L ($P = 0.014$), and HBV-DNA ⩾ 3.7 LGE/mL ($P = 0.008$). Relative risks for HCC were 2.04 (95% CI: 1.10-4.68), 2.00 (95% CI: 1.08-4.56), and 2.84 (95% CI: 1.27-6.34) for these respective factors[1].

It was found by multivariate analysis using these factors that ALT and HBV-DNA levels were independent significant risk factors for HCC. The risk ratio in patients with ALT ⩾ 100 IU/L to that in those with ALT < 100 IU/L was 4.53, while the risk ratio in patients with HBV ⩾ 3.7 LGE/mL to that in those with HBV-DNA < 3.7 LGE/mL was 7.71 (Table 1).

In fact, the interval from suspected diagnosis of cirrhosis to development of HCC in the high-HBV DNA group was shorter than that in the persistently low-HBV DNA group (Figure 1). Moreover, ALT in the high-HBV-DNA titer group was higher than that in the low-HBV-DNA titer group. Despite this finding, serum ALT level was not correlated with HBV-DNA level (Figure 2, $P = 0.353$)[1].

As in our data, Ikeda *et al*[2] reported that the development of HCC in the high-HBV-DNA group was shorter than that in the low-HBV-DNA group. Moreover, they reported that interferon could be effectively administered in a selected group of patients as a strategy for cancer prevention, even in those with cirrhosis. In addition, many reports have revealed that continuous treatment with lamivudine (LAM) delays clinical progression in patients with chronic hepatitis B and advanced fibrosis or cirrhosis, significantly reducing the incidence of hepatic decompensation and the risk of HCC[3,4].

These findings suggest that clinical efforts for viral eradication or reduction of viral load will reduce the risk of HCC in both HBV and HCV-related liver disease. However, in contrast to chronic hepatitis C, the carcinogenesis in chronic hepatitis B in many cases is not correlated with the severity of the fibrotic changes. These epidemiological results suggest that there may be differences in disease activity and mode of disease progression, as well as cancer development, between hepatitis B and hepatitis C viral infections.

HBV-related hepatocarcinogenesis is associated with viral DNA integration into the host genome. Suppression of inflammation and regeneration may reduce the opportunity for DNA integration and, hence, the risk of HCC, although this effect remains to be confirmed in clinical trials[5,6]. Most cases of late-phase recurrence are thought to be due to metachronous multicentric or *de novo* carcinogenesis.

HBV-related hepatocellular carcinogenesis appears to be caused by the hepatitis virus itself rather than the

inflammatory state characteristic of hepatitis. Persistent high HBV-DNA concentration may itself increase the risk of carcinogenesis. Furthermore, clinical and pathological differences exist between HBV genotypes B and C, and genotype C has a higher risk of HCC development than genotype B[7]. Genotype B-related HCC is less associated with liver cirrhosis and has a higher frequency of solitary tumor as well as more satellite nodules than genotype C-related HCC[7].

Sequential analysis of HBV-DNA is the key to assessing the risk of carcinogenesis. HCC develops in younger patients at a higher incidence and more rapidly due to HBV-DNA integration in the early stages of chronic infection.

Previous studies have reported that the incidence of HCC is higher in males than in females, and that the rate of progression of HBV infection is faster in males. As to HBV genotype, on the other hand, no Japanese HCC patients under 35 years in age had the B genotype (genotype Bj), which is different from the B genotype identified in Taiwan (genotype Ba): the genotype Bj is believed to give a relatively good prognosis[8].

Furthermore, HBV-related HCC often occurs in children, and HCC induced by chronic HBV infection also tends to occur much earlier in patients infected in childhood than that in those infected in adulthood.

CLINICAL CHARACTERISTICS OF HBV-RELATED HCC

HBV-related HCC occurs more often in younger patients with mild inflammation and fibrosis than in patients with HCV-related HCC. It has been reported in a domestic multicenter clinical study that liver function is relatively good in patients with HBV-related HCC and they are in disease stage I in many cases, and that the diagnostic age of HBV-related HCC is 52.1 years, while that of HCV-related HCC is 62.9 years, about 10 years later than in HBV-related HCC patients[9].

However, in many cases of HBV-related HCC, the tumor disease stage is advanced (stage Ⅲ/Ⅳ), presenting an undifferentiated cancer type with portal vein infiltration, suggesting that HBV-related HCC has a worse prognosis and a more refractory character than HCV-related HCC. Moreover, HBV-related HCC is infiltrative, showing multinodular growth, unlike HCV-related HCC[10-12].

HBV-related HCC is often accompanied by portal vein tumor thrombus (PVTT). In our study, HBsAg-positive cases were found in 70% of PVTT patients, while anti-HCV serology was positive in 30% of them. Although the prognosis was poor in most patients with HCC accompanied by PVTT, our combination chemotherapy is likely to be effective in achieving long-term survival and appears to exert anti-tumor activities with tolerable adverse effects in patients with advanced HCC accompanied by PVTT[13].

Although recurrence of HBV-related HCC occurs often within 3 years of hepatic resection, the rate of recurrence decreases thereafter. In addition, large tumors are found in many cases of HBV-related HCC, but there are few cases of multicentric recurrence, suggesting that hepatic resection should be the first-choice therapy in such cases[14].

On the other hand, multicentric recurrence occurs at a high incidence after resection in HCV-related HCC patients with impaired liver function, infrequently showing multicentric carcinogenesis at the time of resection in elderly patients, which is a characteristic of HCV-related HCC. Nagasue *et al*[15] reported that the 5-year disease-free survival rate was 58% in patients with HBV infection, whereas it was 6% in those with HCV infection ($P < 0.001$). Thus, major hepatectomy is recommended for treating HBV-related HCC without cirrhosis to prevent early recurrence.

Furthermore, Roayaie *et al*[16] have reported the 5-year disease-free survival rate to be significantly higher in HBV patients treated with resection than in patients with HCV (49% *vs* 7%, $P = 0.0480$). Depending on preoperative liver function and tumor location, a much higher proportion of patients with HBV-related HCC may become candidates for hepatic resection. There are significant differences in preoperative status, tumor characteristics and disease-free survival rate between HCC patients with chronic HBV infection and those with HCV infection whose liver disease has not yet reached the end stage.

In light of these points, curative treatments such as hepatectomy may become the first choice therapy of HBV-related HCC, since many large tumors are found and there are few cases of multicentric carcinogenesis, suggesting that systematic hepatectomy focusing on the morphological type of the portal vein branch may be desirable.

Hepatitis B status, tumor factors, and surgical type affected cancer recurrence after resection in patients with HCC related by concurrent chronic HBV infection, whereas only tumor factors affected recurrence in patients with HCC related by previous chronic HBV infection. Furthermore, Kubo *et al*[14] have reported viral status, defined by a high viral load, the presence of HBeAg, the absence of anti-HBe antibody, and the absence of precore mutant type HBV, as well as low platelet count and positive surgical margin, to be risk factors for recurrence after resection of HCC in HBsAg-positive patients.

In response to these findings, viral elimination has been suggested as a strategy for improving recovery from HBV-related HCC. This prompted Koike *et al*[17] to conduct a univariate analysis, in which they reported that degree of fibrosis, pathological grade of HCC, and serum AFP levels were significantly linked to intrahepatic recurrence. In their multivariate analysis, degree of fibrosis was the strongest factor in the recurrence of HCV-related HCC ($P = 0.004$). In contrast, degree of fibrosis did not influence the recurrence of HBV-related HCC ($P = 0.51$) or NBNC-related ($P = 0.77$) HCC. Risk factors for HCC recurrence varied according to the state of viral infection.

De novo carcinogenesis may be present not only in well-differentiated HCC, but also in less-differentiated HCC, especially in HBV-related HCC; and the carcinogenesis in HBV-related HCC may have different impacts from that in HCV-related HCC. The clinical conditions of hepatitis virus have an impact on the outcomes of resection of HBV-related HCC, making it necessary to position antiviral therapy for prevention of recurrence as one therapeutic option for treating HBV-related HCC.

ANTIVIRAL THERAPY OF HBV-RELATED HCC

Kubo *et al*[18] concluded that the prognosis after resection of HCC was worse in HBeAg-positive patients than in HBeAg-negative patients. Among patients who had undergone transarterial chemolipiodolization, high HBV viral load was the most important risk factor for post-treatment recurrence in cases of complete necrosis, irrespective of the locational pattern of recurrence. The current findings underscore the need for future studies to examine the applicability of antiviral therapy for reducing the risk of HCC recurrence[19].

Recently, LAM, a nucleoside analog that inhibits reverse transcriptase, has been developed. Long-term treatment with LAM has been found to inhibit HBV replication, attenuate hepatitis and improve histological findings of the liver. However, LAM therapy is not free from problems that require resolving, such as relapse of hepatitis as a consequence of the emergence of YMDD viral mutants. The effects of LAM on the therapeutic outcomes of HBV-related HCC patients have not been confirmed in randomized clinical trials (RCTs).

Kubo *et al*[20] have reported that LAM therapy improves the tumor-free survival rate after curative resection in HBV-related HCC patients showing a high serum concentration of HBV DNA, although careful follow-up was necessary for detection of YMDD viral mutants. Kuzuya *et al*[21] reported that there was no significant difference in cumulative recurrence rate of HCC between the LAM group and the control group ($P = 0.622$). However, the median Child-Pugh score at the time of HCC recurrence was significantly different, being 5 (range: 5-6) in the LAM group and 7 (range: 5-12) in the control group ($P = 0.005$).

LAM therapy may be beneficial in patients with HBV-related HCC after initial treatment, since the treatment may contribute to improvement in remnant liver function; therefore, LAM therapy may reduce the risk of liver failure and extend the therapeutic options available for treatment of recurrent HCC.

It is likely that other antiviral therapies such as interferon (IFN) treatment will be effective in HCV-related HCC[22].

Since the HBV-DNA level is a recurrence factor after transcatheter arterial chemoembolization (TACE) in patients with HBV-related HCC, IFN therapy may suppress recurrence and improve convalescence. IFN is believed to suppress tumor recurrence after treatment of HCC in patients with HBV-related cirrhosis, especially in the high-aspartate transaminase (AST) subgroup. In any case, the usability of the prescription of LAM in the following therapy should be reviewed from the viewpoint of versatility. It is expected that the usefulness of LAM will be verified by RCTs, although there is the potential issue of drug tolerance stock.

Therefore, it is likely that LAM will be replaced by entecavir (ETV), the appearance rate of tolerance stock of which is low, as a standard remedy in the future. In this context, it may be necessary to review anew the cancer-suppressive effects as well as the recurrence-suppressive effects of ETV. On the other hand, with respect to HBV-related HCC, IFN was assumed to suppress tumor recurrence after treatment of HCC in patients with HBV-related cirrhosis, especially in the high-AST subgroup[23].

It appears that IFN may suppress recurrence by reducing the viral level or by direct antitumor effects in both HBV-related HCC and HCV-related HCC. Recently, Sun *et al*[24] conducted an RCT, reporting that IFN therapy improved overall survival of patients with HBV-related HCC after curative resection, probably by delay in recurrence.

It will be necessary to review the significance of antiviral therapy in the future, focusing on whether antiviral drugs such as LAM, ETV and IFN can contribute to improved convalescence from HBV-related HCC[25]. Futhermore, LAM will most likely be replaced by tenofovir. Although ETV and pegylated interferon are the other 2 drugs recommended as first-line therapy for HBV, tenofovir remains the first choice. In the future, tenofovir may prove to be effective in preventing recurrence and further investigations involving more cases are needed.

CONCLUSION

Chronic HBV infection is the primary risk factor for the future development of HCC worldwide. Prevention of HBV-related HCC is accomplished by preventing HBV infection *via* HBV vaccination. Broad public health strategies should include routine testing to identify chronic HBV infection, improved health infrastructures including human resources to provide diagnosis and treatment assessment. For persons who are already chronically infected, development of more effective antiviral therapies that can result in sustained suppression of viral replication and hepatic necroinflammation may reduce the incidence of HCC. In the future, it will be necessary to review the significance of antiviral therapy to improve convalescence from HBV-related HCC.

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REVIEW

Current therapeutic strategies for recurrent hepatitis B virus infection after liver transplantation

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Abstract

Hepatitis B virus (HBV)-related liver disease is the leading indication for liver transplantation (LT) in Asia, especially in China. With the introduction of hepatitis B immunoglobulin (HBIG) and oral antiviral drugs, the recurrent HBV infection rate after LT has been evidently reduced. However, complete eradication of recurrent HBV infection after LT is almost impossible. Recurrent graft infection may lead to rapid disease progression and is a frequent cause of death within the first year after LT. At present, the availability of new oral medications, especially nucleoside or nucleotide analogues such as adefovir dipivoxil, entecavir and tenofovir disoproxil fumarate, further strengthens our ability to treat recurrent HBV infection after LT. Moreover, since combined treatment with HBIG and antiviral agents after liver re-transplantation may play an important role in improving the prognosis of recurrent HBV infection, irreversible graft dysfunction secondary to recurrent HBV infection in spite of oral medications should no longer be considered an absolute contraindication for liver re-transplantation. Published reviews focusing on the therapeutic strategies for recurrent HBV infection after LT are very limited. In this article, the current therapeutic strategies for recurrent HBV infection after LT and evolving new trends are reviewed to guide clinical doctors to choose an optimal treatment plan in different clinical settings.

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Key words: Therapy; Hepatitis B virus; Recurrent hepatitis B virus infection; Antiviral drugs; Liver transplantation

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INTRODUCTION

Hepatitis B virus (HBV)-related liver disease accounts for approximately 5%-10% of all liver diseases after liver transplantations (LT) performed each year in the United States and is the leading indication for LT in Asia[1,2]. It was reported that the recurrent HBV infection rate after LT is as high as 80%-100% without any prophylaxis[3,4]. The recurrent HBV infection rate has been decreased from 90% to approximately 30%-40% since the introduction of hepatitis B immunoglobulin (HBIG)[5,6]. With the advances in oral antiviral drugs, combined treatment with HBIG and lamivudine (LAM), recommended by many centers, has achieved encouraging outcomes, with the recurrent HBV infection rate reduced to less than 10% during a follow-up period of 1-2 years[7-11].

However, combined treatment with HBIG and LAM cannot control recurrent HBV infection. Recurrent graft infection may lead to rapid disease progression and is a frequent cause of death within the first year after LT[12].

Furthermore, the incidence of acquiring *de novo* HBV infection after LT in patients who are negative for hepatitis B surface antigen (HBsAg) is 1.7%-3.5%, and patients with *de novo* HBV infection are also at a risk for severe progressive liver injury[13-15]. The aggressive clinical course is probably due to stimulation of viral replication and direct cytotoxicity of HBV under immunosuppressive therapy. Therefore, suppression of HBV replication is paramount to prevent disease progression in the transplanted liver.

Unfortunately, almost all published reviews focusing on the prophylactic strategies against recurrent HBV infection after LT, have drawn less prominence to the treatment of recurrent HBV infection in recipients after LT. Published reviews, focusing on the therapeutic strategies against recurrent HBV infection after LT, are very limited, and almost all of them are already nearly obsolete. In the following, the current therapeutic strategies for recurrent HBV infection after LT and evolving new trends are reviewed.

INTERFERON

In the pre-LAM era, interferon α is a common therapeutic option for patients with recurrent HBV infection after LT. However, with the advent of LAM, it has not been used as a first-line treatment drug. Patients using interferon α have a lower efficacy and a higher risk of precipitating allograft rejection than those using LAM[16,17]. Furthermore, treatment of recurrent HBV infection after LT with interferon α can lead to side effects such as neutropenia.

LAM

LAM can potentially inhibit HBV replication by competitively suppressing the reverse transcriptase and termination of proviral DNA chain extension, and has been used in treatment of recurrent HBV infection, with an excellent safety profile in both compensated and decompensated cirrhotic patients. The use of LAM in treatment of recurrent HBV infection after LT has shown promising results as is shown in a multicenter North American study on 52 patients with chronic hepatitis B after LT, demonstrating that use of LAM for 52 wk can result in loss of serum HBV DNA in 60%, undetectable hepatitis B e antigen (HBeAg) in 31%, undetectable HBsAg in 6%, normalization of serum alanine transaminase (ALT) levels in 71% of patients, respectively[18]. The results from other studies[19-27] are summarized in Table 1, showing that LAM can suppress HBV DNA to undetectable levels in 32.5%-100%, anti-HBeAg seroconversion in 4.2%-100%, and anti-HBsAg seroconversion in 0%-83.3% of patients, respectively, after 4.6-36 mo of treatment. Notably, use of LAM in treatment of *de novo* HBV infection or acute recurrent HBV infection of the graft after LT tends to effectively suppress HBV DNA, and converse serum anti-HBeAg and anti-HBsAg.

However, the major factor limiting the use of LAM in treatment of graft HBV infection after LT is the development of mutations in thyrosine-methionine-aspartate-aspartate (YMDD) motif of the HBV DNA polymerase gene, which confers resistance to LAM. In non-immunosuppressed patients, the LAM resistance rate is 15%-20%[28]. LAM resistance can be detected in 45% of immunosuppressed patients within the first year of treatment[29,30]. It has been reported that YMDD mutation occurs in 26.9%, 27.3%, 29.4% and 62.5% of patients with recurrent HBV infection[18,20,22,24] after 12, 15, 21 and 36 mo of treatment with LAM, respectively. It has also been reported that YMDD mutation occurs in patients with *de novo* HBV infection after LT, in 0%, 0% and 14.3% of patients with recurrent HBV infection[19,21,27] after 4.6, 11 and 24.5 mo of treatment with LAM, respectively. One possible explanation for it is the short-term use of LAM in patients with *de novo* HBV infection and low HBV-DNA levels at the acute phase of *de novo* HBV reactivation.

Available data from the literature regarding the outcomes secondary to YMDD mutation are controversial. Perrillo *et al*[18] reported that the YMDD mutant virus is not consistently associated with hepatic disease progression, whereas elevated serum ALT can be frequently observed at the time when serum HBV DNA becomes detectable again, and the mean ALT values after breakthrough often remain below the pretreatment values. However, McCaughan *et al*[31] reported that patients infected with procure mutant strains of HBV develop drug resistance or liver failure after 11 mo of rescue therapy with LAM. Mutimer *et al*[32] also demonstrated that LAM-resistant phenotype can cause severe graft damage. Hence, long-term, randomized, blinded, and controlled clinical trials are needed to further observe the clinical outcomes following LAM resistance.

In summary, LAM therapy results in not only a loss of viral replication markers in serum and an improved hepatic biochemical profile, but also improvement or stabilization in liver histology. However, LAM resistance and its possible accompanying clinical deterioration have limited its long-term use in treatment of recurrent HBV infection after LT.

Adefovir dipivoxil

Adefovir dipivoxil (ADV), a nucleotide analog that selectively inhibits viral polymerases and reverse transcriptase, is effective against both negative and positive HBeAg[33-36]. Furthermore, it has been shown that ADV has an excellent activity against wild-type as well as LAM-resistant HBV strains[37-41], suggesting that ADV contributes to the development of LAM-resistant mutants and *de novo* LAM-resistant HBV infection after LT. Schiff *et al*[42] in a landmark multicenter study showed that PCR DNA levels are decreased with a 1-year survival rate of 93%, undetectable serum HBV DNA, normal serum ALT, albumin, bilirubin and prothrombin time in 34%, 49%, 76%, 75% and 20% of patients, respectively, after 24 and 48 wk of treatment with ADV. Four years later, Schiff *et al*[43] further showed that the serum HBV DNA

Table 1 Use of LAM in treatment of recurrent HBV graft infection after LT

Author (Ref.)	*n*	Pre-treatment		Treatment duration (mo), mean (range)	LAM dosage (mg/d)	Post-treatment			
		HBV DNA+ (%)	HBeAg+ (%)			HBV DNA negative following treatment (%)	HBeAg seroconversion (%)	HBsAg seroconversion (%)	Development of LAM resistant mutants (%)
Ben-Ari *et al*[20]	8	100	62.5	36 (24-50)	100	32.5	20	0	62.5
Umeda *et al*[19]	6[1]	100	NA	4.6 (0.7-11)	100	NA	NA	83.3	0
Castells *et al*[21]	7[1]	100	85.7	24.5 (12-49)	100	71.4	50	14.3	14.3
Fontana *et al*[22]	33	94	75	21 (4-36)	NA	72	4.2	0	29.4
Andreone *et al*[23]	11[2]	100	18.2	17 (8-27)	100	100	100	9.1	27.3
Perrillo *et al*[18]	52	90.4	86.5	12	100	68.1	11.1	3.8	26.9
Nery *et al*[24]	11	90.9	NA	15 (13-21)	NA	90	NA	NA	27.3
Fischer *et al*[25]	12	100	NA	10.5 (5-43)	100-150	83.3	NA	NA	NA
Rayes *et al*[26]	41	100	NA	12-36	150	75.6	NA	NA	24.4
Malkan *et al*[27]	4[1]	100	75	11 (4-28)	100-150	100	NA	25	0

[1]All patients had *de novo* HBV infection after LT; [2]This study reported treatment of acute recurrent graft HBV infection. NA: Not available; HBV: Hepatitis B virus; LT: Liver transplantation; HBeAg: Hepatitis B e antigen; HBsAg: Hepatitis B surface antigen; LAM: Lamivudine.

Table 2 Use of ADV in treatment of recurrent graft infection after LT

Author (Ref.)	*n*	Pre-treatment		Treatment duration (mo), mean (range)	ADV dosage (mg/d)	Concurrent LAM use	Post-treatment			
		HBV DNA+ (%)	HBeAg+ (%)				HBV DNA negative following treatment (%)	HBsAg seroconversion (%)	ALT normalization (%)	Development of ADV mutants
Akyildiz *et al*[44]	11	81.8	9.1	18 (6-48)	10	Yes	77.8	11.1	81.8	None
Limquiaco *et al*[45]	7	100	71.4	35 (22-48)	10	Yes	28.6	20	86	None
Bárcena *et al*[46]	42	100	71.4	21.5 (12-31)	10	No	64	20	70.5	None
Herreros de Tejada Echanojáuregui *et al*[47]	7	100	71.4	11	10	Yes[1]	42.9	20	NA	None
Neff *et al*[48]	9	100	77.8	30 (6-48)	10	No	0	57.1	NA	None

[1]LAM was changed to ADV in 5 patients and ADV was added to LAM in the other 2 patients. ADV: Adefovir dipivoxil.

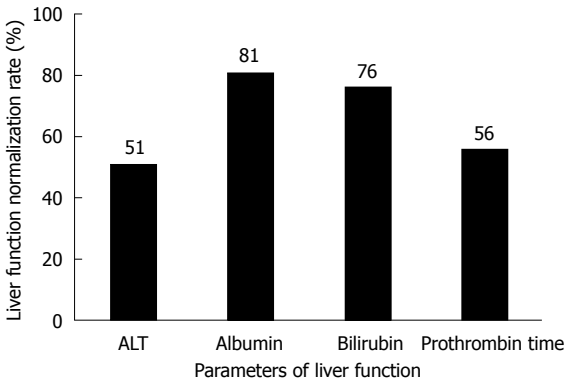


Figure 1 Alanine transaminase (ALT), albumin and bilirubin levels and prothrombin time 48 wk after adefovir dipivoxil treatment[43].

levels are undetectable in 40%, 65% and 78% of patients with recurrent HBV infection and LAM resistance 48, 96 and 114 wk after ADV treatment (10 mg, once a day), respectively, as well as loss or seroconversion of HBeAg in 31% and 11% of patients by week 48, and in 58% and 34% of patients by week 96. Liver function and other parameters after treatment with ADV are shown in Figure 1. Other studies[44-48] are summarized in Table 2. Toniutto *et al*[38] reported that ADV plus LAM can achieve favorable outcomes of HBsAg seroconversion and undetectable HBV DNA in patients with *de novo* graft HBV infection and LAM resistance.

Mildly elevated serum creatinine level may occur after treatment with ADV, especially with calcineurin inhibitors, but only a small number of patients require dose adjustment, and even discontinuance. However, renal function should be regularly monitored, with dose adjustments based on renal function, as necessary.

In short, ADV is a safe and effective treatment option for recurrent HBV infection, especially as a salvage treatment for recurrent HBV infection due to LAM resistance.

Entecavir

Entecavir (ETV), a very potent anti-HBV selective guanosine analogue, approved by the US FDA in 2005, can be used in treatment of chronic HBV infection. Unfortunately, few reports are available on ETV in treatment of recurrent HBV infection. Most data concerning its efficacy and safety are obtained from patients with no LT. It was reported that 0.5 mg of ETV or 100 mg of LAM, once daily for 48 wk, can improve the histology

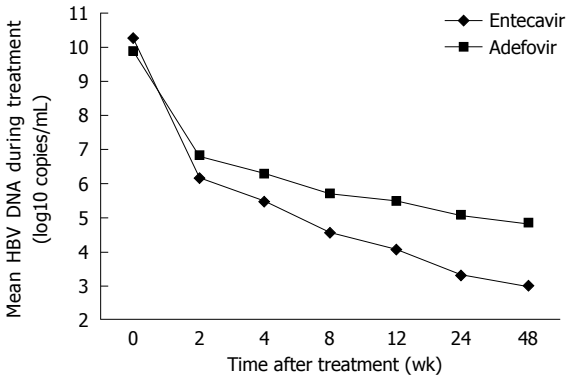


Figure 2 Mean serum hepatitis B virus (HBV) DNA level in patients after entecavir (ETV) and adefovir (ADV) treatment[53].

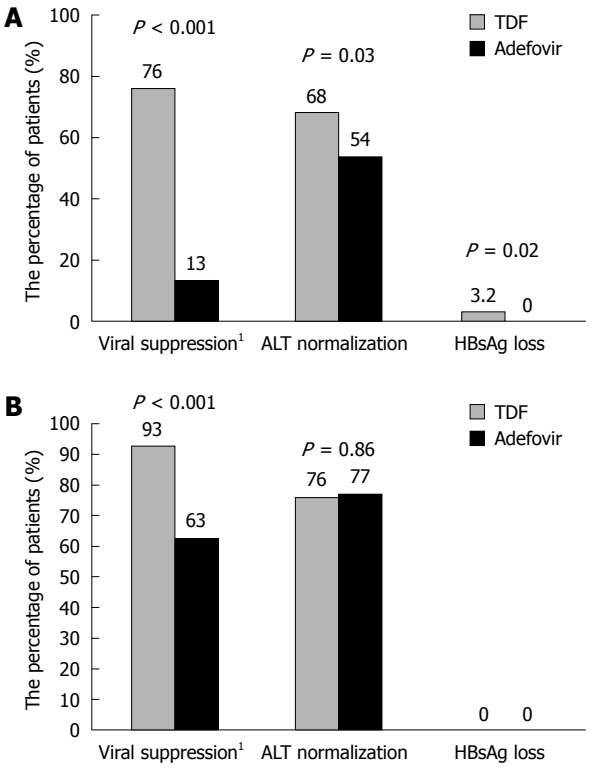


Figure 3 Viral suppression, ALT normalization and hepatitis B surface antigen (HBsAg) loss in hepatitis B e antigen (HBeAg)-positive (A) and HBeAg-negative (B) patients after tenofovir disoproxil fumarate (TDF) and adefovir treatment[61]. [1]HBV DNA level of less than 400 copies/mL.

in 72% and 62% of patients, respectively, with serum HBV DNA undetectable in 67% and 36% of patients, respectively, and normal ALT in 68% and 60% of patients, respectively, and no evidence of viral resistance to ETV[49]. It has been shown that 0.5 mg of ETV or 100 mg of LAM, once daily for 48 wk, can also achieve favorable outcomes in patients with nucleoside-naive HBeAg-negative chronic hepatitis B[50], suggesting that the histologic, virologic and biochemical improvement rates are significantly higher after ETV treatment than after LAM treatment, in patients with nucleoside-naive HBeAg-positive or -negative chronic hepatitis B, with no evidence of viral resistance to ETV, which is consistent with the findings in other studies[51-53]. The mean decrease in serum HBV DNA after 2, 4, 8, 12, 24 and 48 wk of ETV and ADV treatment in patients is shown in Figure 2. A recent study demonstrated that ETV therapy is safe and efficient for recipients with ADV-resistant HBV infection[54].

ETV resistance is associated with the LAM-resistance substitutions M204V/I and L180M in combination with an additional substitution at residues T184, S202 or M250 in the reverse-transcriptase region of HBV polymerase[55]. In other words, ETV is associated with a high genetic barrier to resistance requiring multiple mutations for resistance development. In nucleoside-naïve patients, the probability of developing resistance to ETV remains consistently low (< 1.2%) even after 96 wk of treatment[56]. By contrast, ETV gives rise to ETV-resistant mutants in patients with LAM resistance. The rate of ETV resistance in LAM-resistant patients 4 years after treatment of may reach 35%[57], which is due to a particular selection mode of ETV strains that follows a 2-step process, with the selection of primary resistance mutations at position M204V/I (which are also resistant to LAM) followed by the addition of secondary resistance mutations on the same viral genomes[58]. Once these secondary substitutions occur, high-level resistance to ETV occurs. Therefore, the high probability of resistance to long-term ETV in LAM resistant patients with no LT suggests that ETV is not a good choice for LAM-resistant patients after LT, although ETV has been tried in some LAM resistant patients after LT[54]. However, ETV can be used in non-LAM resistant patients, due to its great potency, high genetic barrier and absence of nephrotoxicity. More data are urgently needed to confirm the safety and efficacy of ETV after LT.

Tenofovir disoproxil fumarate

Tenofovir disoproxil fumarate (TDF), a nucleotide analogue, inhibits viral polymerases by direct binding to DNA or by terminating the DNA chain due to the absence of a requisite 3' hydroxyl on the tenofovir molecule[59]. In 2001, TDF was licensed for HIV therapy as a potent inhibitor of HIV replication. Subsequently, it has been proved to be a potent and selective anti-HBV agent *in vitro*[60]. Several clinical studies have currently confirmed the efficacy of TDF in suppressing HBV replication. Marcellin *et al*[61] showed that the antiviral efficacy of TDF is higher than that of ADV with no resistance mutation in patients with HBeAg-negative or HBeAg-positive chronic HBV infection. Detail data regarding viral suppression, liver function improvement, and serologic response are shown in Figure 3. It has been shown that TDF has an excellent antiviral activity against both wild-type and LAM-resistant HBV both *in vitro* and *in vivo*[62,63]. Furthermore, TDF shows a stronger antiviral effect than ADV on LAM-resistant HBV[64,65]. Hann *et al*[66] reported that the mean level of HBV DNA is 1.5 ± 1.0 log10 copies/mL and 4.3 ± 2.2 log10 copies/mL, respectively, in patients with LAM

resistance after treatment with TDF and ADV. A recent study demonstrated that TDF plus LAM can safely and markedly suppress HBV replication in patients with or without resistance to ADV[67].

The daily TDF dose (300 mg) has been approved as a standard dose for controlling HBV infection. To reduce the cost of therapy, a low TDF dose (75 mg) can be used to control HBV viremia in patients with chronic HBeAg-negative hepatitis B, which can more effectively inhibit HBV replication than ADV at the standard dose[68]. However, further studies are needed to determine its efficacy and safety in a larger number of HBeAg-positive subjects.

Only 2 reports are available on TDF-related mutations conferring resistance to TDF[69,70]. Transfection experiments showed that rtA194T mutation alone can result in a 7.6-fold decrease in susceptibility to TDF, but rtA194T mutation in combination with ADV-resistant mutations can lead to a more than 10-fold decrease in susceptibility to TDF[70]. However, TDF susceptibility to rtA194T mutation is not consistent with its clinical significance. Since 2005, ADV-resistance mutations (rtN236T, rtA194T, rtA181V) have not caused a significant change in TDF susceptibility[71,72]. Only one study is available on the application of TDF in treatment of recurrent HBV infection after LT[73]. Although TDF can significantly decrease LAM-resistant HBV variant replication after LT, further studies are needed to determine its efficacy and safety profile with a long follow-up time and a large cohort of patients.

RETRANSPLANTATION

With the advent of new antiviral drugs such as ADV, ETV and TDF, satisfactory outcomes can be achieved in patients with recurrent HBV infection by suppressing viral replication. However, some patients with irreversible graft dysfunction secondary to recurrent HBV infection still need a second LT to replace the primary graft[74,75]. To date, second LT accounts for 5%-22% of all LTs in adults[76]. Samuel *et al*[77] reported that recurrent graft HBV infection is the direct cause of death in patients with hepatitis B after a second LT. Crippin *et al*[78] showed that the mortality rate of patients with recurrent HBV infection is 95% after a second LT, which is consistent with the findings in other studies[12,79-81].

Ishitani *et al*[82] showed that long-term HBIG therapy can continuously maintain a serum anti-HBs level > 500 IU/L and prevent recurrent HBV infection in 86% of patients after a second LT. Roche *et al*[80] reported that prophylactic intravenous ganciclovir can maintain an anti-HBs titre greater than 500 IU/L, with a satisfactory prognosis of all patients after a second LT. Lo *et al*[83] demonstrated that combined injection of ADV, LAM and HBIG can maintain an anti-HBs titre greater than 100 IU/L in patients with LAM-resistant HBV mutants after a second LT.

In summary, recurrent HBV infection after a primary LT should no longer be considered an absolute contraindication for a second LT. Combined HBIG and antiviral agents may play an important role in improving the prognosis of patients with recurrent HBV infection after a second LT. Further randomized study is needed to definitely confirm it.

Table 3 Summary of recommended therapeutic strategies for recurrent HBV infection after LT

Clinical setting	Recommended first line therapies	Recommended second line therapies
De novo HBV infection, wild-type HBV infection	LAM for short-term therapy; ADV for long-term therapy	ETV, TDF
LAM resistance	ADV, ADV + LAM	TDF
ADV resistance	ETV	TDF + LAM
Irreversible graft dysfunction	SLT	

ETV: Entecavir; TDF: Tenofovir disoproxil fumarate; SLT: Second liver transplantation.

CONCLUSION

The emergence of new oral medications, especially nucleoside or nucleotide analogues such as ADV, ETV and TDF, further increases our ability to treat recurrent HBV infection after LT. By recognizing the efficacies and features of such drugs, various therapeutic strategies, according to different clinical settings, have been recommended to control the recurrent graft infection after LT (Table 3). For the *de novo* or wild-type HBV infection after LT, long-term use of LAM is limited by the high risk of developing mutations in YMDD motif. Hence, ADV is the first choice in this setting, if needing long-term administration. For the LAM-resistant HBV infection after LT, ADV, administered in combination with LAM or as a monotherapy, appears to be safe and effective in this setting. Theoretically, "add therapy" may minimize the risk of resistance, but needs long-term, randomized, blinded, controlled clinical trials to further confirm it. In addition, ETV is not a good choice for LAM-resistant patients after LT because of its high probability of resistance in patients with LAM resistance without LT. ETV therapy for ADV-resistant HBV infection after LT is safe and efficient in this setting. Additionally, combined TDF and LAM therapy may be an alternative approach based on its favorable therapeutic effects in patients without LT. However, no or few data are available on the efficacy of ETV and TDF in patients after LT. Further clinical trials are needed to evaluate their efficacy and safety in this special setting. Second LT is the only rescue procedure for patients with irreversible graft dysfunction secondary to recurrent HBV infection. Since combined HBIG and antiviral agents may play an important role in improving the prognosis of patients with recurrent HBV infection after LT, recurrent HBV infection after a primary LT should no longer be considered an absolute contraindication for a second LT.

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ORIGINAL ARTICLE

AMACR is associated with advanced pathologic risk factors in sporadic colorectal adenomas

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Abstract

AIM: To analyze α-methylacyl CoA racemase (AMACR) expression in relation to various dysplasia phenotypes and clinicopathological parameters of sporadic colorectal adenomas.

METHODS: Fifty-five cases of sporadic colorectal adenomas were categorized according to the Vienna classification for Gastrointestinal Neoplasia. These corresponded to a total of 98 different intra-lesion microscopic fields that were further independently assigned a histological grade based on the old nomenclature (mild, moderate, severe dyplasia and carcinoma *in situ*). AMACR expression was evaluated by immunohistochemistry and statistical analysis was performed to investigate possible associations with various clinicopathologic parameters of adenomas i.e. gender, age, localization, grade of dysplasia, size and configuration.

RESULTS: Patient age ranged from 41 to 84 years (mean 65 ± 13.2 years); 37 patients were males and 18 were females. Adenomas ranged in size between 0.5 and 30 cm (mean 2 ± 1.3 cm), including 18 tubular, 16 villous, 20 mixed or tubulovillous, and 1 giant sessile villous adenoma. AMACR expression was observed in 3 out of 16 (18.8%) of low-grade *vs* 23 out of 35 (62.8%) of high-grade lesions (P = 0.002). Most adenomas exhibiting high grade dysplasia with *in situ* carcinoma-like areas stained positive for AMACR (15/17 or 88.2%) as compared to adenomas with high grade dysplasia which contained severe dysplasia-like foci (6/15 or 40%), (P = 0.005). In AMACR positive adenomas featuring severe dysplasia-like or *in situ* carcinoma-like areas, AMACR staining was not necessarily observed in the *in situ* component. Positivity in intra-lesion of mild, moderate or severe dysplasia-like foci was more often encountered in adenomas harboring *in situ*, intramucosal or infiltrative carcinoma [21/33 (63.6%) *vs* 9/40 (22.5%), P < 0.001]. Strong AMACR expression was found in 11 out of 17 villous adenomas, but in only 1 out of 18 tubular lesions (P = 0.005). Larger lesions, i.e. > 1 cm stained more frequently for AMACR than smaller ones [27/45 (60%) *vs* 2/10 (20%), P = 0.02]. Overall, AMACR expression was associated with the grade of dysplasia, as well as with the size and configuration of adenomas, i.e. the consensus risk factors applied to colorectal adenoma patient surveillance.

CONCLUSION: It may be worthy to further evaluate the possible use of AMACR as an additional risk factor for the assessment of colorectal adenoma patients.

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Key words: α-methylacyl CoA racemase; Adenoma; Colorectal; Dysplasia; Carcinoma *in situ*; Immunohistochemistry

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INTRODUCTION

α-methylacyl CoA racemase (AMACR) is a mitochondrial and peroxisomal enzyme implicated in the degradation of branched chained fatty acids and fatty acid derivatives. Conversion of several (2R)-methyl-branched-chain fatty acyl-coenzyme-A esters to their (S)-stereoisomeres, which is catalyzed by AMACR, is necessary for the completion of the β-oxidation pathway[1-3]. AMACR is expressed in a variety of tumors and precancerous conditions[4-6], with prostate cancer being the one most extensively investigated[7,8]. Various studies have analyzed AMACR expression in adenocarcinoma of the colon, where it is thought to be associated with tumor differentiation and localization[9-14]. Recently, much attention has been given to the expression of this biomarker in preneoplastic conditions arising in the gastrointestinal tract. It has been suggested that AMACR could be used as an adjunct in the characterization of dysplasia in Crohn's disease, ulcerative colitis and Barrett's esophagous[15-17]. In spite of all this promising data, there is still limited information regarding AMACR expression in relation to clinicopathologic parameters as well as to various dysplasia phenotypes in sporadic colorectal adenomas[5,6,10,11,17]. In the paper by Went *et al*[5], no significant differences were found between mild, moderate and severe dysplasia. On the contrary, Strater *et al*[17] found moderate to strong AMACR expression in 73% of high-grade cases but only in 37% of low-grade lesions. However, the results of these studies appear contradictory; in addition, given the degree of interobserver variability in regard to dysplasia classification, interpretation of these findings is hindered by the absence of criteria upon which dysplasia of adenomas was categorized[5,17]. Current guidelines on the classification of dysplasia in sporadic adenomas of the colorectum, namely those published by the American College of Gastroenterology and included in the Vienna classification of Gastrointestinal Neoplasia, strongly discourage the use of terms such as carcinoma *in situ*/intramucosal carcinoma due to, among other reasons, concerns for misinterpretation of their clinical significance that might lead to overtreatment[18-23]. Nonetheless, albeit prone to interobserver variability, specific cytoarchitectural features, including mild, moderate, severe and carcinoma *in situ* changes, do correspond to the biologic progression of dysplasia[20,23]. The goal of our study was to analyze the immunohistochemical expression of AMACR in various histologic patterns of dysplasia in sporadic colorectal adenomas in the frame of clinically oriented current classification schemes.

MATERIALS AND METHODS

Case selection

Fifty-five cases of colorectal adenomas including 4 adenomatous polyps having undergone malignant transformation with submucosal invasion were retrieved from the surgical pathology archives at "Georgios Gennimatas" General Community Hospital from 2005 to 2009. All 55 cases came from individual patients. Out of these, 51 were endoscopically or surgically removed adenomatous polyps and 4 were incidental findings in patients who had undergone colectomy for adenocarcinoma of the colorectum. Clinical data were reviewed to ensure that all cases were indeed sporadic adenomas. Hyperplastic polyps and serrated adenomas were not included in this study based on evidence of their distinguishing biology[24-26]. Hematoxylin-eosin (HE) stained slides were reviewed by two different pathologists in a blinded manner. For diagnostic purposes, each case was characterized by the highest grade of dysplasia present, according to the modified Vienna classification of Gastrointestinal Neoplasia using both the main categories (classical Western classification) as well as the subdivisions of group 4 (high-grade dysplasia)[19,21]. In addition, when more than one dysplastic pattern was histologically recognized on the same slide, these were given a separate grade of dysplasia corresponding to the presumably different biologic potentials and based upon previously reported pathologic criteria[20,27]. In this context terminology was based on the old nomenclature (mild, moderate, severe dysplasia, carcinoma *in situ* and intramucosal carcinoma). Interobserver disagreement on the interpretation of histology was present in 5 cases and a consensus diagnosis was reached at a multi-headed microscope. Size, architectural configuration and localization of lesions were also recorded. Normal appearing non-neoplastic intestinal mucosa was present in 29 cases.

Immunohistochemistry

One 4 micron formalin-fixed, paraffin-embedded tissue section including the highest grade of dysplasia was selected for each case. Prostate cancer sections were used as a positive control. Immunohistochemistry was carried out on a Bond Automated Immunostainer using the Bond Polymer Define Detection Kit (Cat. No. DS9713). Briefly, sections were deparaffinized, rehydrated and treated with 3% H_2O_2 to block endogenous peroxidase activity. Antigen retrieval was performed at 100℃/pH 6 for 30 min. After incubation with the primary polyclonal antibody to AMACR (P504S) (Biocare Medical, Concord, USA) at 1:30 dilution for 30 min, sections were further incubated with a secondary Polymer Poly-HRP anti-mouse/rabbit IgG. 3,3'-diaminobenzidine was used

Table 1 Incidence of various dysplasia histological phenotypes *n* (%)

Clinically oriented	Biologic potential	Total cases	Subgroups
Low grade dysplasia	Mild	16 (29.1)	6 (10.9)
	Moderate		10 (18.2)
High grade dysplasia	Severe	35 (63.6)	15 (27.3)
	In situ carcinoma		17 (30.9)
	Intramucosal carcinoma		3 (5.5)
Infiltrative carcinoma	Submucosal carcinoma	4 (7.3)	

as a substrate for visualization. Finally, slides were counterstained with hematoxylin.

Interpretation

A lesion was considered negative when less than 5% of cells showed immunoreactivity. Positivity was divided into weak (mild, cytoplasmic granular staining) and strong (diffuse, intense cytoplasmic staining). Intra-lesion areas with different grades of dysplasia were evaluated separately, but case-specific intensity was based on the interpretation of immunostaining in the most intense focus, independently of the corresponding dysplastic grade. For example, adenomas containing strongly positive severe dysplasia and negative carcinoma *in situ* foci were considered positive (case 52 in Figure 1A).

Statistical analysis

Statistical analysis was performed using the SPSS software v.17. A P value of < 0.05 was considered statistically significant as determined by the Mann-Whitney test for two independent samples.

RESULTS

Clinicopathological features of colorectal adenomas

Patient age ranged from 41 to 84 years (mean 65 ± 13.2 years); 37 patients were males and 18 were females. Adenomas ranged in size between 0.5 and 30 cm (mean 2 ± 1.3 cm, whereby the largest lesion corresponded to a giant sessile, villous adenoma), including 18 tubular, 17 villous (containing a villous component > 75%), 20 mixed or tubulovillous (containing a villous component > 25%, but < 75%). The majority were retrieved from the left colon with only 7 cases being located in the proximal 2/3 of the transverse colon. The incidence of dysplastic lesions based on the Vienna classification is summarized in Table 1. The well established interrelations between size, histological configuration of colorectal adenomas and grade of dysplasia[28] were reproduced by our data. High grade dysplasia was assigned to 33 out of 41 adenomas greater than 1 cm and 30 out of 34 containing a villous component. In contrast the majority of smaller (7/10) or tubular lesions (12/17) were low grade adenomas. ($P = 0.004$ and $P < 0.001$, respectively). Similarly, lesions ⩾ 1 cm were more likely to contain villous features [31/41 (75.6%) *vs* 3/10 (30%), $P = 0.007$]. Not surprisingly the 3 out of 4 high grade polyps showing evidence of submucosal invasion were villous adenomas and all 4 measured more than 1 cm.

Table 2 AMACR expression in sporadic colorectal adenomas *n* (%)

Vienna classification of GI neoplasia	Biologic potential	Staining				
		Negative	Weak	Strong	Subtotal positive	Total
Low grade ($n = 16$)	Mild moderate	13 (81.3)	3 (18.8)	0 (0)	3 (18.8)	16
High grade ($n = 35$)	Severe	9 (60)	6 (40)	0 (0)	6 (40)	15
	In situ carcinoma	2 (11.8)	3 (17.6)	12 (70.6)[1]	15 (88.2)	17
	Intramucosal carcinoma	1	0	2	2	3
Infiltrative carcinoma ($n = 4$)	Submucosal carcinoma	1	1	2	3	4
		26 (47.3)	13 (23.7)	16 (29)	29 (52.7)	55

[1]In 3 cases (#48, 49, 52 in Figure 1A) higher grade focus and focus of most intense staining did not match, as described in Materials and Methods, Interpretation. AMACR: α-methylacyl CoA racemase; GI: Gastrointestinal.

Immunohistochemical findings

The results of immunohistochemistry are summarized in Table 2 and Figure 1A. AMACR was positive in 29 adenomas (52.7%), and in all 4 adenocarcinomas, but was consistently negative in nonneoplastic intestinal mucosa in all 29 cases where normal appearing colonic epithelium was included in the section. Immunopositivity was strongly related to the grade of dysplasia independent of staining intensity (Figure 1A-C). Twenty-three out of 35 high grade but only 3/16 low grade lesions showed immunopositivity for AMACR ($P = 0.002$). Moreover, when staining was compared among adenomas containing only severe dysplasia-like areas and those with carcinoma *in situ*-like morphology, the latter group was more often positive [6/15 (40%) *vs* 15/17 (88.2%), $P = 0.005$] (Figure 1B and C).

As shown in Figure 1A, with the exception of 3 cases (#48, 49, 52), the most intense AMACR staining was observed in areas with the highest degree of dysplasia. On the contrary, no significant difference in staining intensity was observed between adenomas with mild to moderate dysplasia *vs* adenomas with severe dysplasia (3/16 *vs* 6/15, $P = 0.2$). AMACR immunostaining was also found to be associated with size and configuration of adenomas. Adenomas with a villous component (villous or tubulovillous) were more often positive for AMACR [22/37 (59.5%) *vs* 7/18 (37.5%), $P = 0.03$] and the same applied to lesions measuring ⩾ 1 cm in greatest diameter [27/45 (60%) *vs* 2/10 (20%), $P = 0.02$]. Moreover, as shown in Table 3, strong expression was found in more than half of villous adenomas (11/17), but in only 1 tubular lesion ($P = 0.005$).

When areas with different degrees of dysplasia (and, hence, putative different biologic potential) within the same lesion were separately evaluated for AMACR expression ($n = 98$), positive staining in intra-lesion mild,

Table 3 AMACR expression in relation to pathological features of adenomas *n* (%)

	n	Staining			
		Negative	Weak	Strong	Total positivity
Configuration	55				
Tubular	18	11 (61)	6 (33)	1 (6)	7 (39)
Villous	17	5 (29.4)	1 (5.9)	11 (64.7)	12 (76.5)
Tubulovillous	20	10 (50)	6 (30)	4 (20)	10 (50)
Size	55				
< 1 cm	10	8 (80)	1 (10)	1 (10)	2 (20)
⩾ 1 cm	45	18 (40)	12 (26.7)	15 (33.3)	27 (60)

Table 4 AMACR staining in areas with mild/moderate or severe dysplasia *n* (%)

Higher grade component	Staining			
	Negative	Weak	Strong	Total positivity
Present (*n* = 33)	12 (36.4)	11 (33.3)	10 (30.3)	21 (63.6)
Absent (*n* = 40)	31 (77.5)	9 (22.5)	0 (0)	9 (22.5)

moderate or severe dysplasia-like foci was more frequently encountered in adenomas also harboring a higher grade focus, i.e. *in situ*, intramucosal or infiltrative carcinoma, [21/33 (63.6%) *vs* 9/40 (22.5%), $P < 0.001$], (Table 4 and Figure 1A). Interestingly strong AMACR expression in 9 foci with severe dysplasia and in 1 focus with mild/moderate dysplasia was restricted in adenomas exhibiting intra-lesion cytological and architectural features of a higher grade component in another field (Figure 1A, cases # 43, 47-53, 55). This intra-lesion heterogeneity of AMACR expression is exemplified in Figure 2.

However, when considering severe dysplasia-like and *in situ* carcinoma-like foci as individual cases (that is, not taking into account their occurrence within the same or in different lesions), we did not observe significant differences in AMACR expression ($P = 0.06$). Similarly, no association was found between AMACR expression and either age, gender or localization of adenomas (data not shown).

DISCUSSION

Colonic adenomas are considered adenocarcinoma precursors[29]. Even though this might not be entirely endorsed[30], the predictive value of high grade dysplasia for the subsequent development of advanced adenomas and cancer is well documented and indisputable[31-34]. In this setting, it is widely accepted that severe dysplasia and carcinoma *in situ* bear a similar clinical significance and are therefore merged into a single category in most classification/reporting protocols. Molecular studies have shown an increased frequency of genetic alterations and DNA aneuploidy with increasing grade of dysplasia as assigned by the Vienna classification[35]. Following a detailed evaluation of the examined dysplastic lesions and taking into account intra-lesion heterogeneity, our study is the first one to report a positive association between AMACR and increasing grade of dysplasia in sporadic colorectal adenomas at the immunohistochemical level.

The observed overall prevalence of AMACR expression in colorectal adenomas in our study (52.7%) appears to be somewhat lower than in previous publications (64%-91%)[5,6,10,11,17]. It was difficult to compare our findings with the ones presented in these studies, in which the applied criteria for the classification of dysplasia were not provided. In the two studies that, to our knowledge, evaluated AMACR expression in relation to dysplasia, results did not significantly differ between low and high grade adenomas[17] or among adenomas with mild, moderate or severe dysplasia[5]. The above discrepancies can be attributed to the different antibodies used in each case and also to the different evaluation criteria set for immunopositivity, such as lower thresholds. These differences are further reflected in the reported positivity of normal colonic epithelia[5,11,14,17], which was not observed in our series.

It was previously suggested that AMACR expression might be closely related to the early stages of neoplastic progression in the colon[10]. The estimated prevalence of AMACR in adenomas bearing *in situ* carcinoma cytoarchitectural features in our study (88.2%) is similar to that reported for colorectal carcinoma in the majority of publications (62%-83%)[5,9-12]. In comparison, we observed AMACR positivity in only 40% of severely dysplastic and 18.8% of low grade lesions. These findings might suggest a more advanced state towards malignancy of *in situ* carcinoma as compared to severe dysplasia, although both lesions are currently incorporated into the high grade category for management reasons.

The differences observed between lesions exhibiting morphological changes corresponding to the previously used terms of severe dysplasia and *in situ* carcinoma at the molecular level prompt for morphologically identifying these lesions at least for research purposes[20,21]. Of note, however, co-existing severely dysplastic and *in situ* carcinoma areas in the same lesion did not significantly differ in terms of AMACR expression. AMACR expression seemed to be related to the co-existence of *in situ* carcinoma rather than to *in situ* carcinoma itself, since it was present in severely dysplastic areas in the vicinity of negative *in situ* carcinomas. Severe dysplasia-like morphology does not differ in the simultaneous presence or absence of adjacent *in situ* carcinoma. However, the same morphologic features might bear significant differences at the molecular level that seem to be more prominent in later stages of the dysplasia-carcinoma sequence.

Another implication of the above described intra-lesion AMACR heterogeneity is that, when examining the expression of AMACR and possibly other markers in adenomas on tissue microarrays, multiple areas should be sampled for adequate evaluation of the lesion.

Taken together, our data indicate that AMACR may

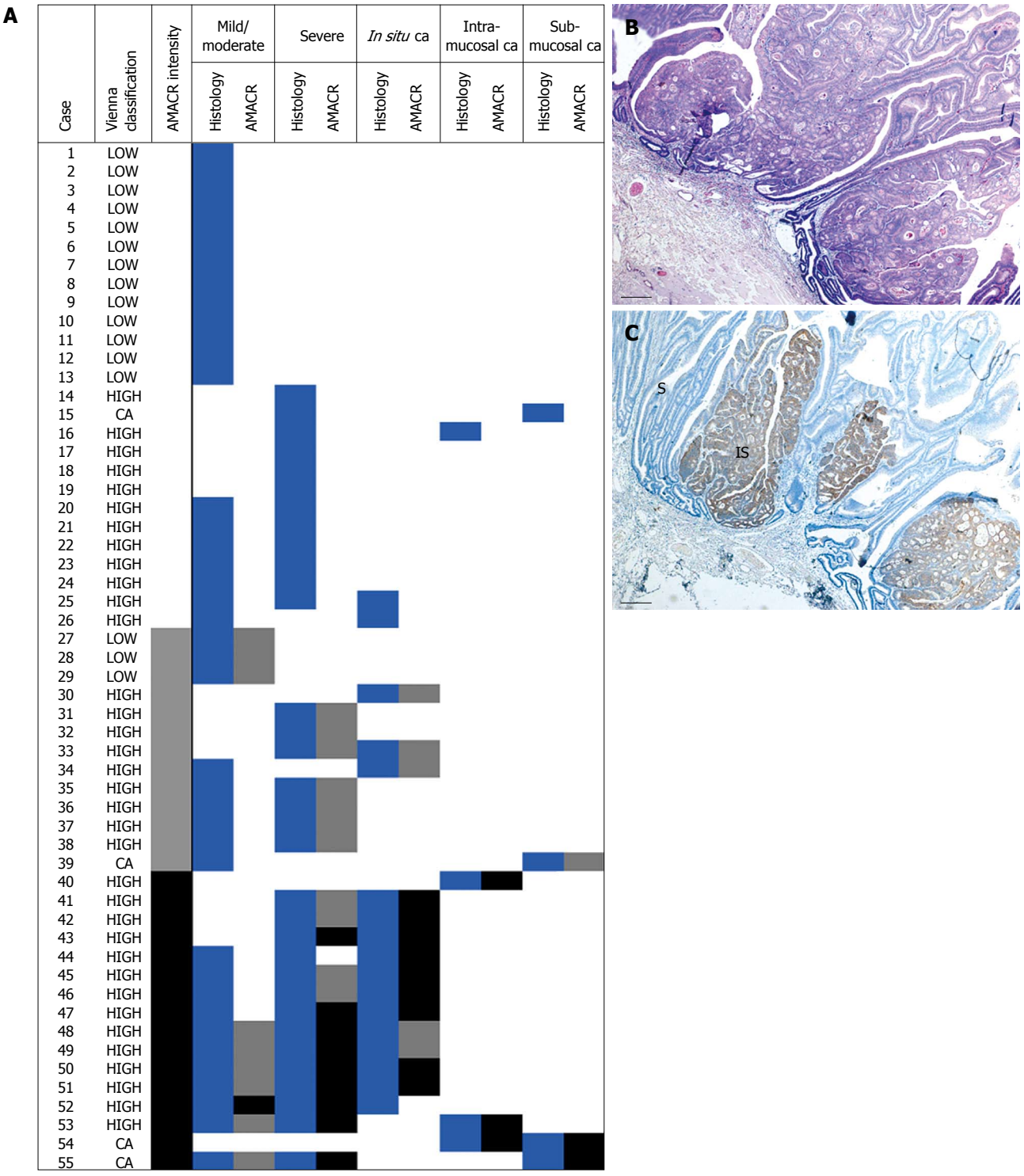


Figure 1 **α-methylacyl CoA racemase (AMACR) expression in colorectal adenomas is related to the grade of dysplasia.** A: Cumulative results from all dysplastic areas within each one of the 55 cases examined. Histology columns: white = absence, blue = presence of the indicated histological pattern. AMACR column: white = negative, grey = weak, black = strong staining; B and C: Case 44, as listed in A. In this adenoma with high grade dysplasia (HE staining), AMACR staining is restricted to the *in situ* component (IS) with severely dysplastic (S) adjacent glands showing negative or focal weak immunoreactivity. Bars: 100 microns.

play some role in the process of malignant transformation in the colon. What this role might be cannot be established by this study. As has also been suggested for AMACR in the prostate, it is uncertain whether its expression is directly involved in the process of malignant transformation or whether it is just an epiphenomenon triggered by the increased metabolic requirements of malignant cells[36].

Studies like the present one are performed to approach a basic question: How can molecular or genetic information help to stratify risk in patients with adenomatous polyps[33]. Currently, size ⩾ 1 cm, villous config-

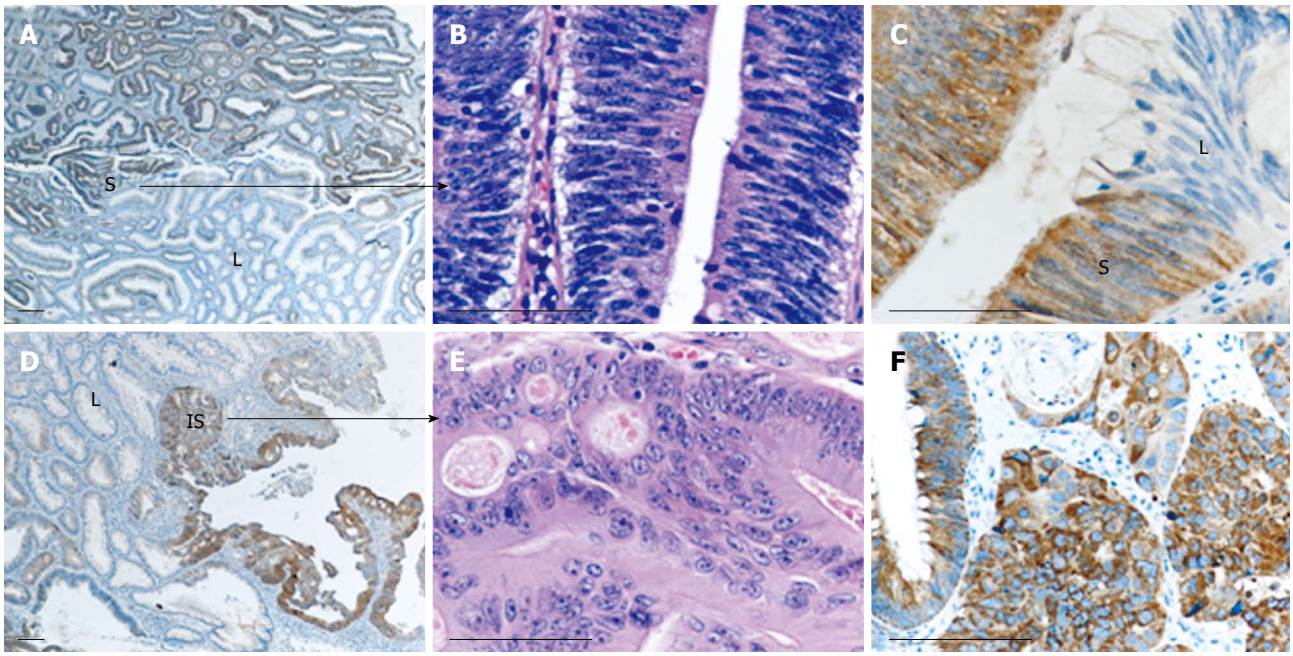


Figure 2 Heterogeneity of AMACR staining may be observed in different dysplastic areas within the same lesion. Case 47, as listed in Figure 1A, is shown. This case contained areas of mild/moderate [low-grade (L)] and severe (S) dysplasia, as well as carcinoma *in situ* (IS) elements. Severely dysplastic (A-C) and carcinoma *in situ* areas (D-F) are strongly positive for AMACR, while mild/moderate dysplastic areas are negative. Histological distinction between severe dysplasia and carcinoma *in situ* is apparent in B and E, respectively (HE staining). Bars: 100 microns.

uration and high grade dysplasia constitute the consensus criteria based on which an adenoma is characterized as "advanced" and patient surveillance is adjusted accordingly[18,31,33,34]. Based on these parameters, patients can be stratified at the time of colonoscopy into lower or higher risk groups for subsequent advanced neoplasia (adenomas with high grade dysplasia or cancer). However, debate on the clinical relevance of these factors continues to exist and many authors still address the need for more objective and standardized pathologic criteria[37-40]. As we show here, except for its association with the grade of dysplasia, AMACR expression in colonic adenomas is also related to two additional pathologic risk factors, i.e. size and villous configuration, a finding not previously reported. Hence, AMACR could be a candidate parameter for further evaluation as an additional risk factor for the development of subsequent advanced neoplasia. Large scale studies with long term patient follow-up would be required for the evaluation of any marker in this setting, since patients with adenoma seldom develop carcinoma within a period of 3 years[32].

Another observation that needs to be further evaluated is the significant difference in AMACR expression between severe dysplasia-like changes and carcinoma *in situ*-like changes, when these appear as independent lesions. Taking into account the drawbacks of our study (small sample size and lack of patient follow up), none of our findings directly raises any issues concerning the current guidelines for the reporting of dysplasia in colorectal adenomas that have been imposed by the need for simplicity and standardization. However as shown here a more detailed approach to the cytoarchitectural features of dysplasia in the context of observational studies might unravel meaningful and potentially useful associations.

COMMENTS

Background

α-methylacyl CoA racemase (AMACR) has been widely used in prostate pathology as an adjunct in the differential diagnosis of prostate cancer. Recently many papers have addressed the possible application of this biomarker in distinguishing low-grade from high-grade dysplasia in premalignant lesions of the gastrointestinal tract like Barrett's esophagus, Crohn's disease and ulcerative colitis.

Research frontiers

Although immunohistochemistry with AMACR in colorectal carcinoma has been extensively investigated, there are not yet any clear-cut conclusions regarding the expression of AMACR in sporadic colorectal adenomas in relation to histology. This is the first study to report associations between AMACR expression and cytoarchitectural features of colorectal polyps.

Innovations and breakthroughs

Recent studies have shown a positive association between AMACR expression and grade of dysplasia in inflammatory bowel disease and Barrett's esophagus with higher grade lesions showing strong staining at the immunohistochemical level. In this study, the authors report for the first time a significant association between AMACR expression and grade of dysplasia in sporadic colorectal adenomas, which may also be extended to other pathologic parameters like villousness and size.

Applications

AMACR may be considered as a candidate adjunct marker for further evaluation regarding risk stratification of patients undergoing colonoscopic surveillance.

Terminology

AMACR is a mitochondrial and peroxisomal enzyme implicated in the degradation of branched chained fatty acids and fatty acid derivatives. There are no conclusive results about the role of AMACR in carcinogenesis. However, multiple studies have addressed its potential use as a biomarker in cancer diagnostics.

Peer review

The study evaluate the expression of AMACR with polyps characteristics and suggested that it could be use as an additional risk factor for the assessment of colorectal adenoma.

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ORIGINAL ARTICLE

Lifestyle-related disease in Crohn's disease: Relapse prevention by a semi-vegetarian diet

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Abstract

AIM: To investigate whether semi-vegetarian diet (SVD) has a preventive effect against relapse of Crohn's disease (CD) in patients who have achieved remission, who are a high-risk group for relapse.

METHODS: A prospective, single center, 2-year clinical trial was conducted. Twenty-two adult CD patients who achieved clinical remission either medically (n = 17) or surgically (n = 5) and consumed an SVD during hospitalization were advised to continue with an SVD and avoid known high-risk foods for inflammatory bowel disease. The primary endpoint was clinical relapse defined as the appearance of active symptoms of CD. Kaplan-Meier survival analysis was used to calculate the cumulative proportion of patients who had a relapse. A 2-year analysis of relapse rates of patients who followed an SVD and those who did not (an omnivorous diet group) was undertaken.

RESULTS: SVD was continued by 16 patients (compliance 73%). Remission was maintained in 15 of 16 patients (94%) in the SVD group *vs* two of six (33%) in the omnivorous group. Remission rate with SVD was 100% at 1 year and 92% at 2 years. SVD showed significant prevention in the time to relapse compared to that in the omnivorous group (P = 0.0003, log rank test). The concentration of C-reactive protein was normal at the final visit in more than half of the patients in remission who were taking an SVD, who maintained remission during the study (9/15; 60%), who terminated follow-up (8/12; 67%), and who completed 2 years follow-up (7/10; 70%). There was no untoward effect of SVD.

CONCLUSION: SVD was highly effective in preventing relapse in CD.

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Key words: Crohn's disease; Inflammatory bowel disease; Vegetarian diet; Recurrence; Lifestyle

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INTRODUCTION

The etiology of inflammatory bowel disease (IBD), which is a collective term for Crohn's disease (CD) and ulcerative colitis (UC), is unknown but is believed to be

multifactorial, whereby development of the disease in genetically susceptible subjects is triggered by environmental factors[1]. IBD is a complex polygenic disease in which the contribution of each gene to the onset of disease is small[2]. IBD incidence increases along with wealth[3,4]. The above two main features of IBD are exactly the same as those for other chronic diseases such as diabetes mellitus, coronary heart disease, and obesity in developed countries[5]. A large part of chronic diseases is related to lifestyle, including food and being sedentary. Some of the environmental factors that have been observed in association with IBD include cigarette smoking, the use of oral contraceptives, and appendectomy[3]. However, these environmental factors are thought to play only a mediating role in IBD. A real key environmental factor has not been identified. Consequently, there is no particular recommendation on lifestyle in guidelines except for smoking in CD[6-9].

Recent studies have clarified that the presence of gut indigenous microflora is a prerequisite for gut inflammation[10,11], and susceptible IBD genes identified are thought to play a role in recognition of microbial agents, immunoregulation, and inflammation[2]. Therefore, IBD is thought to result from an inappropriate response of the mucosal immune system to the intestinal microflora in a genetically susceptible individual[2]. An imbalance of gut microflora, a decrease in beneficial (preventive) bacteria, and an increase in potentially pathogenic bacteria, called dysbiosis, is observed in patients with IBD[12-14]. Dysbiosis can be a trigger for onset and relapse of IBD[15].

There is enough evidence to indicate that IBD is a diet-related disease. Epidemiology shows that IBD is prevalent in wealthy nations[3,4,16] where dietary westernization inevitably occurs[17,18]. Dietary westernization is characterized by increased consumption of animal protein, animal fat, and sugar, with decreased consumption of grains. A dietary study during dietary transition in Japan incriminated an increased intake of animal fat and animal protein in the increase in CD[19]. Pre-illness case-control studies, including those in Japan, have reported increased intake of sugar[20-23], fast foods, chocolate, and cola drinks in IBD[24,25], and a decrease in total fruit and vegetable fiber in CD[20,26]. Case-control studies in Japan are consistent; western foods including bread are a risk factor, whereas traditional Japanese foods are a preventive factor[27-29]. The same tendency has recently been reported in pediatric CD cases in Canada: a positive association with a western diet (meats, fatty foods, and desserts) and an inverse association with a prudent diet (vegetables, fruits, olive oil, grains, and nuts)[30]. Diets rich in animal protein and animal fat cause a decrease in beneficial bacteria in the intestine[31,32]. Probiotics that consist of beneficial bacteria can prevent and are effective against pouchitis, which is ileal inflammation after proctocolectomy in UC[33,34]. These pieces of evidence are consistent with the notion that a westernized diet is linked to an increase in IBD. Therefore, we regard IBD as a lifestyle-related disease that is mediated by mainly a westernized diet[35,36]. Consequently, if a suitable diet is identified and patients stick to the diet, we believe that the majority of IBD patients could be free from relapse without medication.

The conventional recommended diet for IBD is a low-residue diet[37]. A fear of irritating the bowel with dietary fiber has led to a low-residue diet. However, there is no evidence that such a diet is ideal for IBD. A low-residue diet that lacks non-digestible carbohydrates might accelerate the dysbiosis in IBD. Meals *per se* are thought to cause gut inflammation. Therefore, about half of the daily energy intake is provided by an elemental diet, which is a standard regimen in quiescent CD in Japan[38,39]. At present, it is unknown what kind of diet is suitable for IBD.

Therefore, we designed a diet that hopefully increases the number of beneficial bacteria. Limited foods are known to increase beneficial bacteria: green tea and unrefined brown rice[40,41]. However, most prebiotics are extracts of plants[42]. Therefore, we thought that a vegetarian diet would be suitable for IBD. Considering that excessive restriction in foods can be less acceptable, a semi-vegetarian diet (SVD) could be appropriate. Therefore, an SVD has been provided to IBD patients in our hospital since 2003[35,36]. SVD, which is rich in dietary fiber, is quite opposite to conventional low-residue diets in IBD[37].

The biggest problem in practice in IBD is a lack of a safe, long-lasting modality for relapse prevention. Relapse in CD is far more untenable than in UC[43]. The risk of relapse in CD is influenced by the interval of remission: a higher risk among patients with recently achieved remission than among patients in remission[44].

In this study, an SVD was provided throughout the induction phase in CD patients, or after intestinal surgery. After their discharge, they were immediately followed up in the IBD outpatient department. The aim of this study was to investigate whether an SVD has a preventive effect against relapse in high-risk patients who have recently achieved remission.

MATERIALS AND METHODS

Study design

This was a prospective clinical trial with 2 years follow-up to establish whether an SVD has a preventive effect against relapse in high-risk patients who have recently achieved remission. This study was approved by the Ethical Committee of Nakadori General Hospital and all patients gave written informed consent.

Subjects

SVD was first introduced in our institution in 2003, and it has been provided to all patients with CD. From April 2003 to December 2008, 25 active adult CD patients over 19 years old were admitted to the Division of Gastroenterology, Nakadori General Hospital, Akita, Japan.

Table 1 Details of patients' data

Case No.	Sex	Age (yr)	Type of disease	Anal lesion		Disease duration	Previous segmental resection	Initial onset or relapse	Hospitalization						
				Fistula	Tag				On admission		Main medication	Duration of SVD (d)	On discharge		
									CDAI	CRP (mg/dL)			CDAI	CRP (mg/dL)	Morphology
1	F	52	C	-	-	< 1 mo	-	I	161	0.6	Sulfasalazine	58	25	0.9	Active (CS)
2	M	22	C	+	+	4 yr and 3 mo	+	R	264	3.1	Infliximab	55	62	0	NT
3	F	21	EC	+	-	5 yr and 8 mo	-	R	133	6.3	Infliximab	48	38	0	NT
4	M	22	EC	+	-	5 yr and 3 mo	-	R	679	5.9	Infliximab	82	72	0.1	Remission (CS)
5	M	23	EC	+	-	< 2 mo	-	I	235	1.5	Infliximab	55	98	0.1	Near remission (CS)
6	M	28	C	-	-	2 mo	-	I	88	2.5	Infliximab	68	59	0.5	Near remission (CS)
7	F	77	C	-	-	1 yr and 10 mo	-	R	233	13.8	Infliximab	49	39	0	Remission (CS and BE)
8	M	30	C	+	-	6 mo	-	I	281	4.6	Infliximab	50	39	0.1	Remission (CS)
9	M	55	C	+	+	8 mo	-	I	172	1.0	Infliximab	46	67	0.5	Remission (CS and BE)
10	M	19	EC	+	-	4 mo	-	I	147	0.8	Infliximab	43	40	0	Remission (CS and BE)
11	F	21	EC	-	+	3 mo	-	I	335	6.0	Infliximab	49	47	0.3	Improved still active (CS and BE)
12	F	50	E	-	-	1 mo	-	I	488	5.1	Infliximab	43	53	0	Remission (CS)
13	M	28	C	-	-	3 mo	-	I	52	0.6	Infliximab	50	2	0	Remission (CS)
14	M	19	EC	+	+	2 yr and 1 mo	-	I	193	0.8	Infliximab	43	35	0	Near remission (CS and BE)
15	M	29	C	+	-	1 yr and 4 mo	-	I	126	0.2	Infliximab	43	8	0	Improved still active (CS)
16	M	30	C	+	-	1 yr and 1 mo	-	I	211	11.8	Infliximab	47	28	0.8	Near remission (CS and BE)
17	F	21	EC	-	+	6 yr and 2 mo	-	R	544	3.7	Infliximab	46	64	0.3	Improved still active (BE)
18	F	45	EC	-	-	13 yr	+	R, PO 21st d	143	0.2	Metronidazole	19	105	0.1	NT
19	M	21	EC	+	+	2 yr	-	I, PO 13th d	217	1.1	Metronidazole	18	166	0.1	NT
20	M	34	EC	-	-	17 yr	-	R, PO 12th d	146	0	Metronidazole	24	108	0.1	NT
21	M	25	C	-	+	13 yr	-	R, PO 17th d	372	7.1	Metronidazole	21	149	0	NT
22	F	23	EC	-	-	8 yr	-	R, PO 25th d	151	8.0	Metronidazole	83	141	0.8	NT

F: Female; M: Male; EC: Enterocolitis; C: Colitis; E: Enteritis; R: Relapse; I: Initial onset; PO: Postoperative; CDAI: Crohn's disease activity index; CRP: C-reactive protein (normal range ≤ 0.3 mg/dL); SVD: Semi-vegetarian diet; CS: Colonoscopy; BE: Barium enema study; NT: Not tested.

Diagnosis of CD was made by established criteria[45]. Two patients did not achieve remission: one patient was referred to another hospital for surgery, and the other patient was discharged part way through treatment at his request. There were three smokers. Two of them accepted the doctor's advice and stopped smoking after admission. One patient resumed smoking again after discharge, and this patient was excluded from this study. Therefore, 22 patients who achieved clinical remission medically (n = 17, No. 1-17, Table 1) or surgically (n = 5, No. 18-22, Table 1) were included in this study. Clinical remission was defined as the disappearance of active symptoms of CD. Male patients predominated over female patients (14:8). Ages ranged from 19 to 77 years old (median: 26.5 years). The number of patients with enterocolitis, enteritis, and colitis was 11, 1, and 10, respectively. Fourteen patients had perianal fistulas and/or anal tags. Eleven patients with anal fistulas had draining pus. Disease duration of medically treated patients ranged from 1 to 74 mo (median: 8.0 mo). That of surgically treated patients was > 8 years except for case 19, who underwent surgery on his initial visit after 2 years with the disease. Two of the 22 patients underwent previous intestinal resection for CD. Thirteen cases were initial onset and nine were relapses. Crohn's disease activity index (CDAI)[46] on admission in medically treated patients ranged from 52 to 679 (median: 211). Five cases showed CDAI < 150, which is arbitrarily defined to be remission in many studies, but they were definitely suffering from active symptoms. Two cases showed very low CDAI, i.e. 88 in case 6 and 52 in case 13, but they had active symptoms: diarrhea for 2 mo and loose stools and lower abdominal pain for 3 mo, respectively. In surgically treated patients, CDAI score on transfer to our division after surgery was

Table 2 Nutritional data and hemoglobin during hospitalization

Case No.	Dietary pattern before hospitalization	Hospitalization									
		On admission					On discharge				
		BMI (kg/m^2)	Albmin (g/dL)	Chol (mg/dL)	ChE (IU/mL)	Hemoglobin (g/dL)	BMI (kg/m^2)	Albmin (g/dL)	Chol (mg/dL)	ChE (IU/mL)	Hemoglobin (g/dL)
1	Pro-Japanese	21.1	4.7	155	4929	14.8	20.5	4.1	145	4959	13.0
2	Standard	17.2	3.8	140	4059	14.0	17.1	4.5	145	4170	15.5
3	Japanese	17.2	3.7	102	2003	8.3	19.2	4.0	152	4117	11.1
4	Pro-Japanese	18.5	2.8	100	789	8.6	17.9	3.0	116	1876	12.7
5	Pro-western	17.7	4.8	132	5592	15.8	17.0	4.2	135	5057	14.4
6	Pro-western	21.3	3.0	122	2876	10.8	20.7	4.4	192	4321	10.8
7	Pro-Japanese	17.2	2.1	95	1549	7.5	16.8	3.2	177	2633	11.6
8	Pro-western	19.2	3.2	128	3441	12.6	18.3	4.2	114	4911	14.3
9	Japanese	19.7	3.9	190	5094	13.0	18.2	4.1	221	4602	14.1
10	Pro-western	17.6	4.0	147	4505	13.5	17.6	4.5	154	4725	13.7
11	Standard	19.8	3.7	147	4081	9.5	18.4	3.9	125	4837	10.3
12	Standard	24.1	4.4	149	5156	11.0	21.4	4.3	158	6626	10.7
13	Standard	24.4	4.6	151	4599	16.2	22.5	4.4	143	4376	15.1
14	Pro-western	19.0	4.7	167	5722	15.0	18.8	4.4	138	4861	16.0
15	Pro-western	24.1	4.6	188	5755	15.4	22.4	4.5	137	5643	15.8
16	Pro-western	22.5	3.7	138	3757	12.3	21.4	4.4	116	4304	14.3
17	Standard	18.8	3.8	187	2700	10.6	18.1	4.1	209	2798	11.8
18	Pro-Japanese	21.6	4.1	126	7943	10.7	21.9	3.9	129	9098	10.8
19	Pro-western	20.1	4.1	150	3320	11.0	20.1	4.2	152	3426	11.8
20	Polymeric diet	18.1	3.9	100	3512	11.0	17.9	4.1	130	3757	13.4
21	Standard	15.6	3.5	116	3387	10.2	15.6	4.0	138	3870	11.9
22	Pro-Japanese	16.5	3.2	58	2320	9.9	16.0	2.9	78	3355	9.8

Standard: A mixture of Japanese and western diet; BMI: Body mass index; Chol: Total serum cholesterol; ChE: Cholinesterase. Normal range: BMI: 18.5 kg/m^2 ≤- < 25.0 kg/m^2; Albmin: 3.8-5.2 g/dL; Chol: 120-220 mg/dL; ChE: 3200-6500 IU/mL; Hemoglobin: M: 13.8-17.5 g/dL, F: 12.0-15.1 g/dL.

determined. The details such as sex, age, clinical types by disease location, anal lesions, disease duration, initial onset or relapse, and C-reactive protein (CRP) concentration are shown in Table 1.

Medical induction of remission: The main medication was infliximab in all medically treated patients except for case 1. Case 1 was the earliest case in this study with mild symptoms and was treated with sulfasalazine 3 g/d. Metronidazole 750 mg/d was given after admission. Patients received liquid infusion of 1500 mL/d *via* a peripheral vein for about 1 wk, without meals. Meanwhile, morphological studies including colonoscopy or contrast barium enema study, enteroclysis, and esophagogastroduodenoscopy were performed to assess clinical types and stenosis. Laboratory data were also obtained. Then, infliximab (5 mg/kg body weight; Remicade, Centocor, Malvern, PA, USA) was infused over 3 h[47]. Infliximab was further infused at 2 and 6 wk according to the standard recommendation[47]. After about 1 mo of metronidazole administration, it was switched to mesalamine 1.5 g/d or sulfasalazine 2 g/d. After the third infusion of infliximab, patients were discharged. Iron (60 mg/d saccharated ferric oxide) was given to patients with moderately severe anemia, but none of patients received a blood transfusion. No steroid hormone, immunosuppressant, or antibiotic other than metronidazole was administered.

The CDAI score of medically treated patients was significantly decreased from 255 ± 169 (mean ± SD) on admission to 46 ± 24 at week 6 after the first infusion of infliximab, i.e. before the third infusion of infliximab (paired *t* test, $P < 0.0001$, Table 1). The concentration of CRP also significantly decreased from 4.0 ± 3.9 to 0.2 ± 0.3 mg/dL ($P = 0.001$). Morphological studies by colonoscopy and/or contrast barium enema study were performed before discharge in 15 patients (Table 1). Morphological remission was achieved in seven patients. Near remission was achieved in four patients in whom redness and/or a few small aphthoid lesions without ulcer were present. In three patients, active lesions were improved, but active lesions (ulcer) were still present. In case 1, there was no improvement in active lesions. Nutritional data in patients who showed hypoalbuminemia (< 3.8 g/dL, $n = 7$) or hypocholesterolemia (< 120 mg/dL, $n = 3$) on admission were improved to a normal level at week 6: from 3.2 ± 0.6 to 3.9 ± 0.6 g/dL ($P = 0.0086$) and from 99 ± 4 to 148 ± 31 mg/dL, respectively (Table 2). Low serum cholinesterase (< 3200 IU/L, $n = 5$) improved: from 1983 ± 855 to 3149 ± 1039 ($P = 0.0233$). There was little change in body mass index (BMI) at week 6 in patients who showed abnormally low levels (< 18.5 kg/m^2, $n = 5$) on admission: from 17.4 ± 0.2 to 17.5 ± 1.0. Anemia (male < 13.8 g/dL, female < 12.0 g/dL, $n = 11$) improved from 10.7 ± 2.0 to 12.3 ± 1.6 ($P = 0.0058$). Details of individual data are presented in Table 2.

Surgical induction of remission: Five patients, cases 18-22, who had recently undergone intestinal resection for intestinal obstruction, were transferred to our division

Table 3 Lifestyle and dietary habits

	Every day	3-5 times/wk	1-2 times/wk	Rare	None
Smoking (No. of cigarettes/d)	20 ≤	6-19	1-5	Rare	P
Regular exercise	R	R	R		P
Alcohol					P
Eating between meals			P	R	R
Type of diet (D)	Semi-vegetarian (R)	Japanese	Pro-Japanese	Standard/mixed	Pro-western (P)
Food					
Rice	P				
Miso soup	P				
Pulses	R	P			
Vegetables	R	P			
Udon/soba (Japanese noodles)				P	
Ramen (Chinese noodles)			P	R	
Bread (D)	P			R	
Tea, coffee		Canned coffee (P)		R	
(Sugar in tea or coffee) (D)	Large amount (P)	Average amount	Small amount	Rare	None (R)
Juice	P			R	R
Cola/soda					P
Beef			P	R	
Pork/chicken (D)		P		R	
Minced or processed meat			P	R	
Fish				P	
Cheese/butter/margarine			P	R	
Sweets (D)	P			R	R
Ice cream/milk shake				P	
Yoghurt (plain)	R				P
Green tea (D)	R				P
Potatoes/starches (D)	R		P		
Fruits (D)	R				P

P: Your present habit (style); R: Recommended habit (style); D: Drastic alteration recommended.

when they could take solid meals. This occurred on post-operative day 12-15 (Table 1). They took metronidazole 750 mg/d for about 1 mo along with meals[48].

Patients' dietary habits: Patients' dietary habits and lifestyles before onset or relapse of the disease were obtained immediately after admission, prior to providing information about the SVD, by means of a food-frequency questionnaire[49]. The questionnaire included 45 questions that covered almost all foods or food groups in Japan. There was a question about dietary type that listed six types: western, pro-western, standard/mixed, pro-Japanese, Japanese, and SVD. A definition of dietary types was not set. There was a variety of dietary patterns before the onset or relapse of CD: pro-western (n = 8), standard/mixed of western and Japanese diet (n = 6), pro-Japanese (n = 5), and Japanese (n = 2) (Table 2). Case 20 had a 2000 kcal/d polymeric diet without meals. None of our patients' dietary pattern was SVD or a western diet. Based on the questionnaire, a summarized table was made that showed both a patient's hitherto and future recommended lifestyle and dietary habits. Such a table created for case 8 is shown in Table 3. Recommended dietary habits were consistent with SVD in the following. This table was used by the dietitian when giving dietary guidance, and it was given to the patient by the chief investigator (Chiba M) during hospitalization.

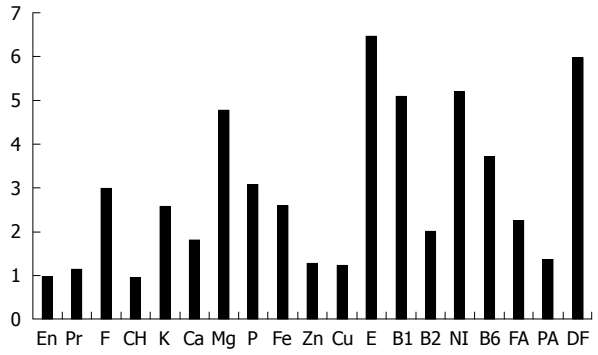


Figure 1 Nutritional elements of brown rice in comparison with white rice[50]. The amount of elements in 100 g of edible food is expressed in comparison with those in white rice, whose value is 1. Brown rice is richer in almost all elements in comparison. En: Energy; Pr: Protein; F: Fat; CH: Carbohydrate; E: Vitamin E; B1: Vitamin B1; B2: Vitamin B2; NI: Niacin; B6: Vitamin B6; FA: Folic acid; PA: Pantothenic acid; DF: Dietary fiber.

SVD: SVD was initiated on the same day as infusion of infliximab in medically treated patients. About 800 or 1100 kcal/d was given in the beginning, and calories were gradually increased to a maximum of about 30 kcal/kg standard body weight. White rice was served first for 2-5 d followed by mixed rice (70% white rice and 30% unrefined whole brown rice) for 2-5 d, and finally brown rice was served. Unrefined brown rice contains more vitamins and minerals than white rice[50] (Figure 1) and is reported to increase significantly beneficial bacteria compared to

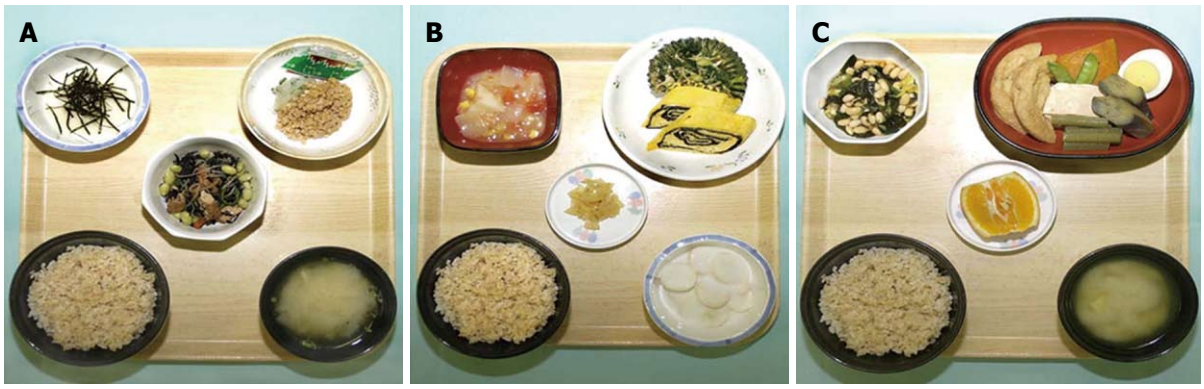


Figure 2 A sample of a 1700-kcal/d semi-vegetarian diet (SVD) for Crohn's disease (CD). From left to right, breakfast (A), lunch (B), and dinner (C). Boiled brown rice is seen at the bottom left of the tray and miso (fermented bean paste) soup is at the bottom right. Breakfast (A): Raw grated nagaimo (yam) and shredded toasted nori seaweed are at the top left; natto (fermented soybeans) and grated daikon (Japanese radish) are at the top right; and braised hijiki seaweed, nama-age (thick deep-fried beancurd), and edamame (young soybeans) are in the center; Lunch (B): Boiled potato, onion, and corn in tomato soup are at the top left; isomaki-tamago (dried nori seaweed inside egg roll) and boiled chrysanthemum with sesame dressing are at the top right; takuan (pickled radish) is in the center; and a mixture of banana and plain yoghurt is at the bottom right; Dinner (C): Boiled chingensai (qing gin cai), soybeans, and wakame seaweed with vinegar soy sauce dressing are at the top left; simmered ganmodoki (bean-curd-based mixture), tofu (bean curd), boiled egg, Japanese-variety eggplant, Japanese butterbur, Japanese-variety pumpkin, and snow peas are at the top right; and citrus fruit is in the center.

Table 4 Content of semi-vegetarian diet (2000 kcal/d)

	SVD	DRI
Energy		
Protein (%)	16.1 ± 0.5	< 20
Fat (%)	18.6 ± 1.4	20-30
Carbohydrate (%)	66.1 ± 1.6	50-70
Content		
Protein (g)	80.3 ± 3.0	M 60, F 50
Fat (g)	41.4 ± 3.6	
Carbohydrate (g)	330.2 ± 11.4	
Dietary fiber (g)	32.4 ± 2.1	M 20, F 17
Soluble dietary fiber (g)	6.8 ± 0.7	
Insoluble dietary fiber (g)	23.3 ± 1.6	
Calcium (mg)	873.7 ± 65.2	M 600-650, F 600
Phosphorus (mg)	1882.7 ± 101.8	M 1050, F 900
Iron (mg)	19.3 ± 1.3	M 7.5, F 10.5
Sodium (mg)	4492.9 ± 342.1	600
Kalium (mg)	4281.7 ± 250.6	M 2000, F 1600
Vitamin A (μgRE)	1416.6 ± 360.9	M 750, F 600
Vitamin B1 (mg)	2.0 ± 0.2	M 1.4, F 1.1
Vitamin B2 (mg)	1.3 ± 0.2	M 1.6, F 1.2
Niacin (mg)	26.6 ± 3.1	M 15, F 12
Vitamin C (mg)	133.1 ± 23.4	100
Vitamin D (μg)	3.2 ± 1.0	5
Vitamin E (mg)	12.3 ± 1.9	M 9, F 8
Cholesterol (mg)	285.9 ± 72.1	M < 700, F < 600
NaCl (g)	10.9 ± 0.8	M < 10, F < 8
Polyunsaturated fatty acid (g)	13.9 ± 1.9	
Monounsaturated fatty acid (g)	9.3 ± 0.9	
Saturated fatty acid (g)	7.1 ± 0.9	
P:S ratio	2.0 ± 0.2	

SVD: Semi-vegetarian diet, Figure is mean ± 1SD of 28 d; DRI: Dietary reference intakes for Japanese[52], Figures for ages above 18 are shown; P:S: Polyunsaturated fatty acids:saturated fatty acids.

well-milled white rice[41]. Eggs and milk were used. In other words, our diet was a lacto-ovo-vegetarian diet[51]. Miso (fermented bean paste) soup, vegetables, fruits, legumes, potatoes, pickled vegetables, and plain yoghurt were served daily. Fish was served once a week and meat once every 2 wk, both at about a half the average amount. Patients were provided with several different 4-wk menus on a rotational basis. Figure 2 shows what an SVD looks like. Details of the contents of nutritional elements including minerals, vitamins, and fatty acids in an SVD are shown in Table 4. These figures were obtained by HOPE/COMETY-NT (Fujitsu, Tokyo, Japan). The rate of fat in total calories (18.6%) was < 20%, which is the lower limit of Dietary Reference Intakes for Japanese (DRI)[52]. The amounts of dietary fiber and iron in the SVD were above the DRI. Those of most of the other elements in the SVD were comparable to DRI (Table 4). Coarse tea was served along with the meal service. During hospitalization, foods other than the meal service were discouraged. Drinking of green tea was encouraged. Participant days of SVD ranged from 43 to 82 d (median: 49 d) in medically treated patients (Table 1). In surgically treated patients, they were shorter, about 3 wk. Case 22 was exceptionally long, 83 d, due to a postoperative complication of subcutaneous abscess formation. In all 22 cases, there were no adverse effects such as gaseous distress, abdominal discomfort, or diarrhea as a result of the SVD.

At the end of hospitalization, a qualified dietitian gave dietary guidance to the patient and the meal preparer. It included how to boil brown rice. The responsible doctor (Chiba M) also gave patients an SVD guide (Figure 3) and advised them to continue the diet after discharge. Foods that have been shown to be a risk factor for IBD in or outside Japan, including sweets[20-23,28-30], bread[27], cheese[27-29], margarine[27], fast foods, carbonated beverages, and juices[24,25,29], were discouraged. Healthy habits were encouraged: no smoking, regular physical activity, moderate or no use of alcohol, regularity of meals, and not eating between meals[53].

Follow-up study

Twenty-two patients who achieved remission either medically or surgically were followed up for 2 years. Medication was mesalamine or sulfasalazine (Table 5). None of

Table 5 Follow-up study

Case No.	Main medication	Dietary pattern				Days of remission	Reason for termination	On final attendance						
		Month 3	Year 1	Year 2	On relapse			CDAI	CRP (mg/dL)	BMI (kg/m^2)	Alb (g/dL)	Chol (mg/dL)	ChE (IU/mL)	Hemoglobin (g/dL)
1	SSZ for 1 yr	SVD	SVD	SVD		730	Completion	0	0.1	23.1	4.5	235	5684	14.4
2	Mesalamine	SVD	Omni		Omni	301	Relapse	193	8.2	17.5	3.8	151	3262	13.8
3	Mesalamine	SVD	Omni		Omni	367	Relapse	167	11.3	21.1	3.5	133	3551	10.0
4	Mesalamine	SVD	SVD	SVD		730	Completion	128	5.0	18.7	2.8	114	1113	9.3
5	SSZ for 1 yr	SVD	SVD			523	Moving	39	0	19.4	4.5	194	5509	15.0
6	Mesalamine	Omni			Omni	191	Relapse	192	5.7	21.7	3.9	134	3052	7.9
7	None	SVD	SVD			442	INFX for RA	61	2.0	19.6	4.0	158	5068	10.9
8	Mesalamine	SVD	SVD		SVD	635	Relapse	174	7.6	19.1	3.1	88	2678	11.3
9	Mesalamine	Omni	Omni	Omni		730	Completion	48	0.2	19.8	4.3	171	5461	15.2
10	Mesalamine	SVD	SVD	SVD		730	Completion	18	0.1	18.2	5.1	181	4659	16.3
11	Mesalamine	SVD	SVD	SVD		730	Completion	11	0	19.7	4.7	147	5215	13.9
12	Mesalamine	SVD	SVD	SVD		730	Completion	45	0	20.2	4.7	163	6268	11.3
13	Mesalamine	SVD	SVD	SVD		730	Completion	0	0	22.8	4.6	171	4770	15.6
14	Mesalamine	SVD	SVD			666	Ongoing	27	0.1	19.3	4.7	188	6084	15.1
15	Mesalamine	SVD	Omni			560	Ongoing	0	0	22.6	4.8	185	5671	16.8
16	Mesalamine	SVD	SVD			440	Ongoing	19	1.9	21.8	4.4	141	5146	14.2
17	Mesalamine	SVD	SVD			397	Ongoing	32	2.0	22.5	3.6	216	2683	11.1
18	Mesalamine	SVD	SVD	SVD		730	Completion	25	0.4	22.6	3.7	151	10222	11.1
19	Mesalamine	SVD	SVD	SVD		730	Completion	62	0	20.1	4.7	139	4391	13.3
20	None	SVD	SVD	SVD		730	Completion	37	0.7	18.2	4.5	156	5655	13.2
21	SSZ	SVD	SVD	SVD		730	Completion	16	0.1	19.0	4.9	205	6424	14.4
22	Mesalamine	SVD	Omni		Omni	630	Relapse	118	5.1	22.1	3.5	84	3596	10.1

Alb: Albumin; SSZ: Sulfasalazine; Omni: Omnivorous diet; INFX: Infliximab; RA: Rheumatoid arthritis. Normal range: CRP: ≤ 0.3 mg/dL; BMI: 18.5 kg/m^2 ≤- < 25.0 kg/m^2; Albmin: 3.8-5.2 g/dL; Chol: 120-220 mg/dL; ChE: 3200-6500 IU/mL; Hemoglobin: M: 13.8-17.5 g/dL, F: 12.0-15.1 g/dL.

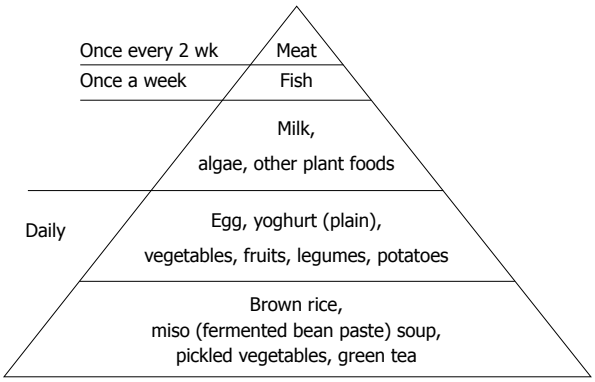


Figure 3 SVD food guide pyramid.

the patients took immunosuppressants such as azathioprine or 6-mercaptopurine. They visited the IBD outpatient department (responsible doctor: Chiba M) every 8 wk. Blood samples for measurement of CRP, complete blood counts, albumin, and transaminases were taken at each visit. The food-frequency questionnaire was obtained at month 3, at years 1 and 2, or when a patient relapsed. When remission was maintained for 1 year and a patient seemed to continue the SVD, medication was stopped if the patient so desired. Primary end point was clinical relapse that was defined as the appearance of active symptoms of CD that required treatment. Duration of remission was calculated as the number of days between discharge and the appearance of symptoms of relapse. Although the responsible doctor (Chiba M) similarly advised patients to continue the SVD, some patients did not follow the advice or abandoned it at some point. Relapse in 2 years was compared between two groups of patients: SVD group and the omnivorous diet group.

Assessment of dietary pattern in outpatients: Dietary pattern was classified into two groups: SVD and omnivorous diet. When the following two conditions were fulfilled, it was regarded as SVD in this study. One is that a patient follows the principle of SVD: daily intake of rice, vegetables, and fruits, and occasional intake of fish, meat, and other animal-based foods. The other one is that a patient refrains from foods reported as risk factors for IBD in or outside Japan as stated above[20-30]. A diet that did not fulfill these two conditions was regarded as an omnivorous diet.

Statistical analysis

Kaplan-Meier survival analysis was used to calculate the cumulative proportion of patients who had a relapse. Patients who stopped the treatment for any reason other than relapse were considered censored at the time of their last observation. Comparison of cumulative relapse rates between the SVD and omnivorous groups was tested by the log rank test. A P value of 0.05 or less was considered to indicate a statistically significant difference. Statistical analysis was performed using JMP 8 (SAS Institute Inc., Cary, NC, USA) software.

RESULTS

Eighteen patients terminated the follow-up: completion

Table 6 Maintenance therapy in Crohn's disease following induction of remission or response

	Induction therapy		Maintenance therapy		
	Subjects	Regimen	Regimen	Duration	
				1 yr	2 yr
Candy *et al*[55], 1995	CDAI > 200	PS (diminishing dose) + AZA 12 wk	AZA 2.5 mg/kg Placebo	Remission rate 42% (14/33) 7% (2/30)	
Hanauer *et al*[56], 2002	CDAI ⩾ 220 Responder to a single infusion of infliximab	Gr (1) and Gr (2) INFX 5 mg/kg at weeks 0, 2, 6 Gr (3) INFX 5 mg/kg then placebo infusion at weeks 2, 6	Gr (1) INFX 5 mg/kg every 8 wk Gr (2) INFX 10 mg/kg every 8 wk Gr (3) placebo infusion every 8 wk	Remission rate 25% (28/113) 33% (37/112) 11% (12/110)	
Sandborn *et al*[58], 2005	Mild to moderate CD	Budesonide 6 mg or 3 mg	Budesonide 6 mg or 3 mg Placebo	Relapse rate 60%-70% (In all 3 groups)	
Takagi *et al*[39], 2006	Active CD	TPN (25 cases), TEN (22 cases), PS (1 case), INFX (3 cases)	ED (half) + a free diet (half) A free diet	Relapse rate 26.9% (7/26) 64.0% (16/25)	
Regueiro *et al*[59], 2009	CD patients who had surgery	Ileocolonic resection	INFX 5 mg/kg at weeks 0, 2, 6 then every 8 wk Placebo	Remission rate 80% (8/10) 58% (7/13)	
Present study	Active CD	INFX 5 mg/kg at weeks 0, 2, 6 (16 cases), intestinal resection (5 cases), SSZ (1 case)	Semi-vegetarian diet Omnivorous diet	Remission rate 100% (16/16) 67% (4/6)	92% 25%

PS: Prednisolone; AZA: Azathioprine; CD: Crohn's disease; TPN: Total parenteral hypernutrition; TEN: Total enteral nutrition; ED: Elemental diet.

of 2-year observation in 11, relapse in five, and early termination for other reason in two (moving and infliximab therapy for associated rheumatoid arthritis in one each). Four patients are ongoing (Table 5). Dietary types that patients marked in the food-frequency questionnaire during the follow-up study were judged appropriate by other foods or food groups marked. SVD was maintained by 16 patients but not by six (compliance 73%) (Table 5). There was no untoward effect of SVD. The reason for the discontinuance of SVD in six patients was not a detrimental effect of SVD but their preference for sweets, meat, and/or fish. Case 2 continued SVD for the first 3 mo but stopped thereafter. Cases 3 and 22 consumed vegetables and fruits only once a week and ate sweets frequently. Case 6 cooked and added meat to the SVD that was prepared by his mother. Case 9 had alcohol at supper every night and frequently ate fish and sweets. Case 15 consumed animal foods more often than the standard for an SVD.

Five patients relapsed. Four were medically treated and one was surgically treated. On the final study visit, they showed elevated CRP concentration and a CDAI of > 100 (Table 5). Seventeen patients maintained remission. Among them, 11 with normal CRP concentration showed almost normal values in other nutritional data (BMI, albumin, cholesterol, and cholinesterase) and hemoglobin. Their CDAI was extremely low. Meanwhile, most of the six patients with elevated CRP, although in remission, showed some abnormality in these indices. The details are shown in Table 5.

Among the 16 patients who continued with the SVD, 15 maintained remission and one relapsed (Table 5): the remission rate was 100% (16/16) at 1 year and 92% at 2 years (Figure 4 and Table 6). Among six patients who were on an omnivorous diet, two maintained remission and four relapsed (Table 5): the remission rate was 67% (4/6) at 1 year and 25% at 2 years (Figure 4 and Table 6). Life table analysis showed that the cumulative relapse rate

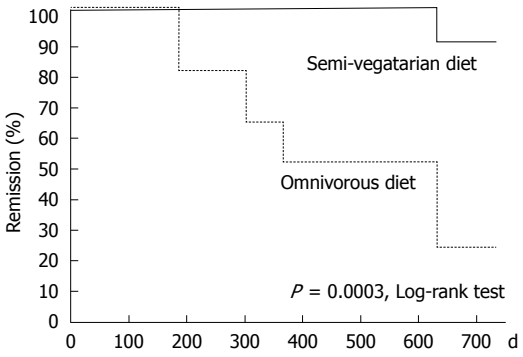


Figure 4 Life table estimate of maintaining remission with SVD or omnivorous diet.

at 2 years was significantly lower in the SVD group than in the omnivorous group (P = 0.0003) (Figure 4). The concentration of CRP was normal at the final visit in more than half of the patients in remission on an SVD; in those who maintained remission during the study (9/15; 60%); terminated follow-up (8/12; 67%), or completed 2 years follow-up (7/10; 70%).

DISCUSSION

We used mesalamine or sulfasalazine in the follow-up phase. Considering that the relapse-preventive effect of mesalamine or sulfasalazine is absent or modest at most[54], it is reasonable to conclude that the SVD protected patients from relapse but an omnivorous diet did not.

Azathioprine and 6-mercaptopurine are known to be effective and used for maintenance of remission in patients with steroid-dependent CD[6-9]. They are also used in the induction phase together with prednisolone and then used for maintenance. In such situations, Candy *et al*[55]

have reported that the remission rate at 1 year was 42% (placebo control, 7%) (Table 6). Recently, scheduled infliximab therapy every 8 wk has been shown to be effective as maintenance treatment in both adults and pediatric patients[56,57]. Remission rate at 1 year in adults was 25%-33% (placebo control, 11%) (Table 6)[56]. An elemental diet that consisted of about a half the daily energy intake has been shown recently to have a relapse-preventive effect in the Japanese study group: 26.9% relapse rate at 1 year (control free diet, 64.0%) (Table 6)[39]. Remission rates with the SVD in the present study were far better than those reported previously: 100% (16/16) remission rate at 1 year and 92% at 2 years. To the best of our knowledge, this is the best result in relapse prevention. Although our study population included mild cases, our excellent results cannot be explained solely by a difference in severity in study population, because even mild to moderate disease relapse rates are 60%-70% at 1 year (Table 6)[58]. More recently, scheduled infliximab therapy every 8 wk has been shown to be effective in postoperative patients: 80% remission rate at 1 year (placebo control, 58%) (Table 6)[59]. Successful scheduled maintenance therapy with infliximab, however, could encounter difficulty over longer periods due to a waning of efficacy, and side effects. In 2 years, more than half the patients need a shortening in the infliximab dosing interval and about 10% of patients need cessation of therapy[60]. Although the number of patients in our study was small, all four postoperative patients on the SVD maintained remission for 2 years (Table 5).

When we began thinking of IBD as a lifestyle-related disease mediated mainly by dietary westernization, we changed our modality of treatment to emphasize diet (Table 7). The menu of a conventional low-residue diet for IBD is shared by UC and CD. However, CD is apparently more tenacious than UC[43]. Therefore, the staple is refined white rice for UC and unrefined brown rice for CD. About 30 kcal/kg per day is provided in the current diet, while total calories were not individualized in the conventional diet. Dietary analysis is needed for dietary guidance in current practice (Table 7). In addition to diet, common health practices[53] are encouraged.

SVD is initiated after about 1 wk of fasting, on the same day as infliximab infusion. By this time, symptoms such as diarrhea, abdominal pain, and fever subside, and patients want to eat. SVD that contained a moderate amount of dietary fiber was not detrimental but useful for induction of remission of CD. It improved hypoalbuminemia, hypocholesterolemia, and anemia during hospitalization. Although clinical remission could be obtained by one or two infusions of infliximab, we believe that a certain period of time is needed for recovery of morphological changes in the intestine. Therefore, three infusions of infliximab in 6 wk[47] were given, not at the outpatient clinic but during hospitalization. This period of hospitalization was also useful for patients to become familiar with the SVD. Compliance with the SVD in outpatients in this study was about 75%, which indicates that an SVD could be applicable to the majority of CD patients. An SVD is completely natural and safe. There is no need to fear side effects, as with steroid hormones or immunosuppressants such as infliximab, azathioprine, 6-mercaptopurine, or methotrexate.

Table 7 Conventional and current diet in IBD at Nakadori General Hospital

	Conventional	Current
Diet	Low-residue diet	Semi-lactoovovegetarian diet
Menu	Common for UC and CD	SVD for UC (staple: white rice) SVD for RUC (staple: white rice 70%, brown rice 30%) SVD for CD (staple: brown rice)
Refrainment	Fiber-rich diet	Minced or processed meat, bread, fast foods, sweets, cola/soda, juices
Dairy products	Refrainment	Egg, milk: no refrainment Refrain from cheese/butter/margarine
Calories	2000 kcal/d	About 30 kcal/kg standard body weight/d
Dietary analysis	Absent	Food-frequency questionnaire
Dietary guidance	Absent	Guidance by doctor and registered dietitian

UC: Ulcerative colitis; RUC: Refractory ulcerative colitis.

Fifteen of 16 patients who continued the SVD were free from relapse in our study. In addition, more than half of the patients in remission showed normal CRP levels. CRP is a sensitive indicator for predicting relapse[61]. Judging from CRP levels, it seems that more than half of the patients who continue the SVD will be free from relapse as long as they maintain the diet. However, the rest of the patients in remission with elevated CRP might relapse in the long term, which seems a limitation of the SVD. Our study clearly shows that the dietary pattern influences the relapse rate in CD. Therefore, trials of the preventive effect of medications in CD should be designed in consideration of diet.

There have been trials for prevention of relapse in CD with diets or supplements. Excellent results with an exclusion diet of intolerant foods[62], an unrefined-carbohydrate, fiber-rich diet[63], or fish oil supplement[64] have not been reproduced in other studies[65-67]. Therefore, none of the dietary modifications has been widely accepted. Currently, manipulation of gut microflora with probiotics and/or prebiotics has emerged as an attractive therapeutic modality in IBD[11,15,33,34]. The rationale of an SVD, i.e. enhancement of beneficial bacteria in the gut, is the same as that of probiotics and prebiotics. There is a limitation to mere addition of probiotics, prebiotics or food stuffs and exclusion of potentially untoward food stuffs[65-68]. We believe that, not partial, but comprehensive food control is needed for patients who are genetically predisposed to IBD. Therefore, our SVD encourages consumption of grains, vegetables, and fruits, while limiting intake of animal foods that tend to decrease beneficial bacteria[31,32] and other foods reported to be risk factors for IBD[20-30]. However, no food item is prohibited.

Although we designed our SVD with gut bacterial flora in mind, both plant-only (vegan) and plant-based (lacto-ovo-vegetarian, semi-vegetarian) vegetarians are shown to have low rates of cancer, cardiovascular disease, obesity, and total mortality[51,69]. Plant-based diets are recommended for prevention of cancer and other lifestyle-related chronic diseases[70]. Therefore, SVD will not only be effective for gut inflammation, but also promote the general health of IBD patients.

Since we are aware that an SVD is effective for relapse prevention, we are concerned with rapid, safe and reliable induction of remission. This is now attainable by infliximab[47]. Therefore, we use infliximab as the first choice of therapy in all newly diagnosed active CD cases. So far, there has been no failure in induction of remission with infliximab (Chiba *et al*, article in preparation). Therefore, since the advent of infliximab and SVD, we feel that CD has been much more controllable than before.

In conclusion, this study shows that an SVD is safe and has a preventive effect against relapse of CD. Normal CRP levels are maintained in more than a half of the patients with an SVD. This supports our notion that IBD is a lifestyle-related disease that is mediated mainly by a westernized diet. The concept that IBD is a lifestyle-related disease is lacking in present practice. We believe that without introduction of this concept, a major breakthrough in the prevention of relapse in CD is not attainable. Whether gut microflora are indeed enriched in beneficial bacteria by an SVD needs to be clarified. Our new findings require verification in large, randomized, controlled clinical trials.

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COMMENTS

Background

Crohn's disease (CD) is a chronic disease of the intestine. The peak incidence is between the ages of 15 and 25 years. Remission is induced by drip infusion or elemental diet (amino-acid-based) without meals, by drugs such as prednisolone and infliximab, by resection of the involved intestine, or by a combination of these. The biggest problem in practice in CD is frequent relapse and difficulty in maintaining remission. At 1 year, the relapse rate is around 60%-70% and the remission rate is 25%-42%. A low-residue diet has been conventionally recommended without evidence of improving remission rates. Because meals inevitably cause relapse, the use of elemental diet is popular in countries like Japan.

Research frontiers

Although the etiology of CD is unknown, there is some evidence to indicate that it is a lifestyle-related disease that is mediated mainly by a westernized diet: increased consumption of animal protein, animal fat, and sugar, with decreased consumption of grains. Therefore, the authors designed a semi-vegetarian diet (SVD) which hopefully increases beneficial (preventive) bacteria in the intestine. In this study, the authors examined whether the SVD had a preventive effect against relapse in high-risk patients with CD who had recently achieved remission.

Innovations and breakthroughs

The SVD was highly effective in preventing relapse in CD. Remission rate with the SVD was 100% at 1 year and 92% at 2 years. This is the best result in relapse prevention. The concentration of C-reactive protein, an indicator of inflammation, was normal at the final visit in more than half of the patients on the SVD, which indicated that more than half of the patients who continue the SVD will be free from relapse as long as they maintain the diet.

Applications

SVDs can be provided anywhere in the world. Ulcerative colitis (UC) is another chronic inflammatory bowel disease that displays a less harsh clinical course than CD. An SVD can also be applied to UC.

Terminology

The SVD in this study was a lacto-ovo-vegetarian diet in which eggs and milk were used. Fish was served once a week and meat once every 2 wk, both at about half of the average amount.

Peer review

This is an excellent paper with clear scientific data in a clinical area of extreme importance.

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ORIGINAL ARTICLE

Histopathology and TB-PCR kit analysis in differentiating the diagnosis of intestinal tuberculosis and Crohn's disease

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Abstract

AIM: To compare the histopathologic features of intestinal tuberculosis (ITB) and Crohn's disease (CD) and to identify whether polymerase chain reaction for *Mycobacterium tuberculosis* (TB-PCR) would be helpful for differential diagnosis between ITB and CD.

METHODS: We selected 97 patients with established diagnoses (55 cases of ITB and 42 cases of CD) who underwent colonoscopic biopsies. Microscopic features of ITB and CD were reviewed, and eight pathologic parameters were evaluated. Nine cases of acid fast bacilli culture-positive specimens and 10 normal colonic tissue specimens were evaluated as the positive and negative control of the TB-PCR test, respectively. PCR assays were done using two commercial kits: kit <A> detected *IS6110* and *MPB64*, and kit <B> detected *IS6110* only; a manual in-house PCR method was also performed on formalin-fixed, paraffin-embedded colonoscopic biopsy specimens.

RESULTS: Statistically significant differences were noted between ITB and CD with regard histopathologic criteria: size of granulomas (P = 0.000), giant cells (P = 0.015), caseation necrosis (P = 0.003), confluent granulomas (P = 0.001), discrete granulomas (P = 0.000), and granulomas with lymphoid cuffs (P = 0.037). However, 29 cases (52.7%) of ITB showed less than five kinds of pathologic parameters, resulting in confusion with CD. The sensitivities and specificities of the TB-PCR test by kit <A>, kit <B>, and the in-house PCR method were 88.9% and 100%, 88.9% and 100%, and 66.7% and 100% in positive and negative controls, respectively. The PCR test done on endoscopic biopsy specimens of ITB and CD were significantly different with kit <A> (P = 0.000) and kit <B> (P = 0.000). The sensitivities and specificities of TB-PCR were 45.5% and 88.1%, 36.4% and 100%, and 5.8% and 100%, for kit <A> and kit <B> and in-house PCR method on endoscopic biopsy specimens. Among the 29 cases of histopathologically confusing CD, 10 cases assayed using kit <A> and 6 cases assayed using kit <B> were TB-PCR positive. A combination of histologic findings and TB-PCR testing led to an increase of diagnostic sensitivity and the increase (from 47.3% to 58.2) was statistically significant with kit <B> (P = 0.000).

CONCLUSION: The TB-PCR test combined with histopathologic factors appears to be a helpful technique in

formulating the differential diagnosis of ITB and CD in endoscopic biopsy samples.

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Key words: Intestine; Tuberculosis; Crohn's disease; Histopathology; Polymerase chain reaction; Kit; *IS6110*; *MPB64*

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INTRODUCTION

Tuberculosis (TB) is one of the most prevalent diseases in the world and a cause of significant mortality and morbidity in developing countries[1]. In Korea, the total number of newly developed TB patients was 19 652 in 2000, but the number was markedly increased to 34 123 in 2001 and 34 157 in 2008[2]. The resurgence of TB is, in part, due to the pandemic of human immunodeficiency virus infection[1,3,4]. The incidence of intestinal TB (ITB), one of the most common forms of extra-pulmonary TB, has been increasing in parallel with the overall resurgence of TB. Sometimes the diagnosis of ITB is very challenging to make, because of a close resemblance to Crohn's disease (CD) in clinical, radiological, endoscopic, and histological appearance[5,6]. This is especially true in Asia, where the incidence and prevalence of CD are also rising[7]. For this reason, definitive diagnosis of ITB and CD is increasingly important. In the case of ITB misdiagnosis, unnecessary anti-tuberculosis therapy poses a risk of toxicity and delays the treatment of CD. Conversely, treatment of ITB with steroids alone can lead to severe deterioration, or even death.

Pathologic features such as size and number of granulomas, giant cells, caseation necrosis, confluent granulomas, discrete granulomas, ulcers with bands of epithelioid histiocytes and granulomas with lymphoid cuffs are known to be important criteria for differentiating ITB and CD[8,9]. Several molecular techniques have been introduced in addition to histopathologic criteria for the detection of mycobacteria in histopathological specimens and for differentiating these two conditions[10-12]. Studies have shown that detection of the *IS6110* gene in *Mycobacterium tuberculosis* (*M. tuberculosis*) through an in-house method has high sensitivity and specificity[13,14], but the results are still controversial. Recently, many kinds of commercial kits have been developed using different targets to detect the *M. tuberculosis* genome, but their diagnostic accuracy has not been sufficiently examined in the literature. In this study, we compared the in-house PCR test, which detects the *IS6110* gene sequence, with two commercially available kits (represented as <A> and <B>) that have been used widely in Korea. Kit <A> detects both the *MPB64* and *IS6110* genes simultaneously, and kit <B> detects the *IS6110* gene sequence only. We reviewed the histopathological features of ITB and CD and performed PCR for *M. tuberculosis* (TB-PCR) in the endoscopic biopsy specimens to evaluate whether the histopathologic features and ITB-PCR would improve the differential diagnosis of these two diseases.

MATERIALS AND METHODS

Patient selection

We reviewed the records of patients who underwent endoscopic biopsies at Inha University Hospital, Incheon, South Korea, between 1996 and 2007 and determined their ITB and CD status. Before the study, an ethical committee approved the study protocol. A total of 334 cases which were pathologically suspicious for chronic colitis were selected. All the clinical data and hematoxylin- and eosin-stained sections were retrieved and reviewed. The definitive diagnosis of ITB or CD was based on clinical symptoms, colonoscopic findings, radiological investigation, pathological evaluation, response to antituberculous treatment, and follow-up data for more than 3 mo. A diagnosis of ITB was made when at least two of the following criteria were met: (1) demonstration of acid-fast bacilli on histologic examination of Ziehl-Neelsen stained sections; (2) positive *M. tuberculosis* culture; (3) radiologic, colonoscopic, and/or operative evidence of ITB with proven tuberculosis elsewhere; and (4) response to antituberculous therapy without subsequent recurrence in patients with radiologic, colonoscopic, and/or operative evidence of ITB. A diagnosis of CD was made when at least two of the following criteria were met: (1) clinical history of abdominal pain, malaise, diarrhea, and/or rectal bleeding; (2) endoscopic findings of mucosal cobblestoning, linear ulceration, skip areas, or perianal disease; (3) presence of enterocutaneous or entero-enteric fistulae and/or chronic perianal disease; and (4) resolution of symptoms and morphological (endoscopic and histological) features after corticosteroid and 5-ASA therapy. Ultimately, a total of 55 cases of ITB and 42 cases of CD were selected and the endoscopic biopsy specimens before medical therapy were used for this study.

Histopathological examination

Retrieved hematoxylin- and eosin-stained sections from ITB and CD patients were reviewed by two pathologists (Jin XJ and Kim JM). They evaluated the eight histopathologic parameters suggested by Pulimood *et al*[8,9]: size

of granuloma (small: < 200 μm, medium: 200-400 μm, large: > 400 μm), numbers of granulomas per biopsy piece, Langerhans giant cells, caseation necrosis (acellular pink areas of necrosis with karyorrhectic debris), confluent granulomas (merging of the adjacent boundaries of granulomas), discrete granulomas (well defined, majority of epithelioid histiocytes conforming to the shape of the granuloma), ulcers with bands of epithelioid histiocytes (conglomerate bands of epithelioid histiocytes lining the ulcer or ulcer slough), and perigranulomatous lymphoid cuffs (lymphoid aggregates surrounding granuloma). We divided the cases into four groups according to histopathologic parameters: group Ⅰ, showing caseation necrosis; group Ⅱ, showing more than four kinds of histopathologic parameters without caseation necrosis; group Ⅲ showing one to four kinds of histopathologic parameters without caseation necrosis; group Ⅳ, without any histopathologic parameters.

Positive and negative control samples for TB-PCR

Nine acid fast bacilli (AFB) culture-positive samples (3 from colon, 3 from bone, 2 from vertebrae, and 1 from a joint) and ten normal colonic mucosa samples obtained from disease-free resection margins of colectomy specimens resected due to colon cancer were used as the positive and negative controls for the TB-PCR test. All samples were open biopsy or resection specimens, enough in sample size and formalin-fixed and paraffin-embedded. DNA extraction and PCR tests were done according to the manual in-house PCR method and instructions of kits <A> and <B>, respectively.

Polymerase chain reaction

DNA extraction: The paraffin-embedded blocks for all samples used in this study were cut at 50 μm thickness and placed in a microcentrifuge tube. For the clinical sample, we used only one block containing one to eight pieces of intestinal mucosa revealing the most representative features of the disease. To prevent carry-over of DNA contamination, the microtome blade was changed after cutting each sample. For deparaffinizing, we added 1 mL xylene, vortexed vigorously, and centrifuged at 14000 r/min for 3 min at room temperature. The pellet was then washed with 100% ethanol twice and air-dried for 15 min at 37℃. Genomic DNA was extracted according to the manufacturer's protocol.

DNA amplification by PCR and detection: For each commercial kit, PCR was accomplished according to the manufacturer's protocols. Briefly, kit <A> was designed to detect the *IS6110* and *MPB64* genes of *M. tuberculosis* simultaneously. The first round of PCR was performed using 3 μL of genomic DNA in a total volume of 20 μL, in the presence of 1 × TB2 first primer, 3 μL of 8-MOP solution, and 1 × multiple master mix. PCR conditions included 35 cycles of 30 s at 95℃, 30 s at 60℃, and 30 s at 72℃. Nested PCR was performed using 3 μL of the first amplification mixture as the template in the same composition of PCR mixture without primer (TB2 second primer instead of TB2 first primer). PCR conditions included 25 cycles of 30 s at 95℃, 30 s at 62℃, and 30 s at 72℃. PCR product size was 520 base pairs (bp) for the internal control, 260 bp for the first PCR product, and 200 bp for the second PCR product. An internal control was used as a standard for success or failure. Kit <B> was designed to detect only the *IS6110* region of *M. tuberculosis*. The first round of PCR was performed using 4.5 μL of genomic DNA with 15.5 μL of first PCR master mix. PCR conditions included 35 cycles of 45 s at 95℃, 45 s at 68℃, and 30 s at 72℃. Nested PCR was performed using 1.5 μL of the first amplification mixture as the template with second PCR master mix. PCR conditions included 25 cycles of 45 s at 95℃, 45 s at 65℃, and 30 s at 72℃. PCR product size was 374 bp for the first PCR product and 158 bp for the second PCR product.

The in-house PCR method used in this study was designed to detect the *IS6110* region of *M. tuberculosis*. The first round of PCR was performed using 5 μL of genomic DNA in a total volume of 25 μL in the presence of 1 × Ex *Taq* buffer (10 mmol/L Tris-HCl, 50 mmol/L KCl, 2 mmol/L $MgCl_2$), 2 U Ex *Taq* DNA polymerase (TAKARA, Japan), and 10 pmol of each primer (KBN-1: 5'-GGATGGTCGCAGAGATCCBGC-3', KBN-2: 5'-CGATGCCCTCACGGTTCAGG-3'). PCR conditions were as follows: 35 cycles of 30 s at 95℃, 1 min at 60℃, and 1 min at 72℃, followed by a final extension step of 10 min at 72℃. For the second round of PCR, 1 μL of the first amplification mixture was used as the template in a total volume of 25 μL containing 10 pmol of each primer (KBN-3: 5'-GTCAGACGATTCGGAGTGG-3', KBN-4: 5'-TGATGATCGGCGATGAACCG-3'). PCR conditions were as follows: 35 cycles of 30 s at 95℃, 1 min at 60℃, and 1 min at 72℃, followed by a final extension step of 10 min at 72℃. PCR product size was 297 bp and was analyzed by gel electrophoresis on 2% agarose gels stained with ethidium bromide.

Statistical analysis

We used the R version 2.9.2 (A free software environment for statistical computing and graphics, Copyright 2009 The R Foundation for Statistical Computing, ISBN 3-9000051-07-0) for analyzing the histopathologic features and PCR results by χ^2 test and Fisher's exact test and for analyzing the increase of diagnostic sensitivity by Logistic regression analysis. *P* value less than 0.05 was considered statistically significant. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated.

RESULTS

Clinicopathologic findings of ITB and CD

Out of a total of 97 cases, 55 cases were ITB, and 42 cases were CD. The mean ages of the ITB and CD patients were 39 and 27 years, respectively. The most common site of involvement for ITB was the right colon

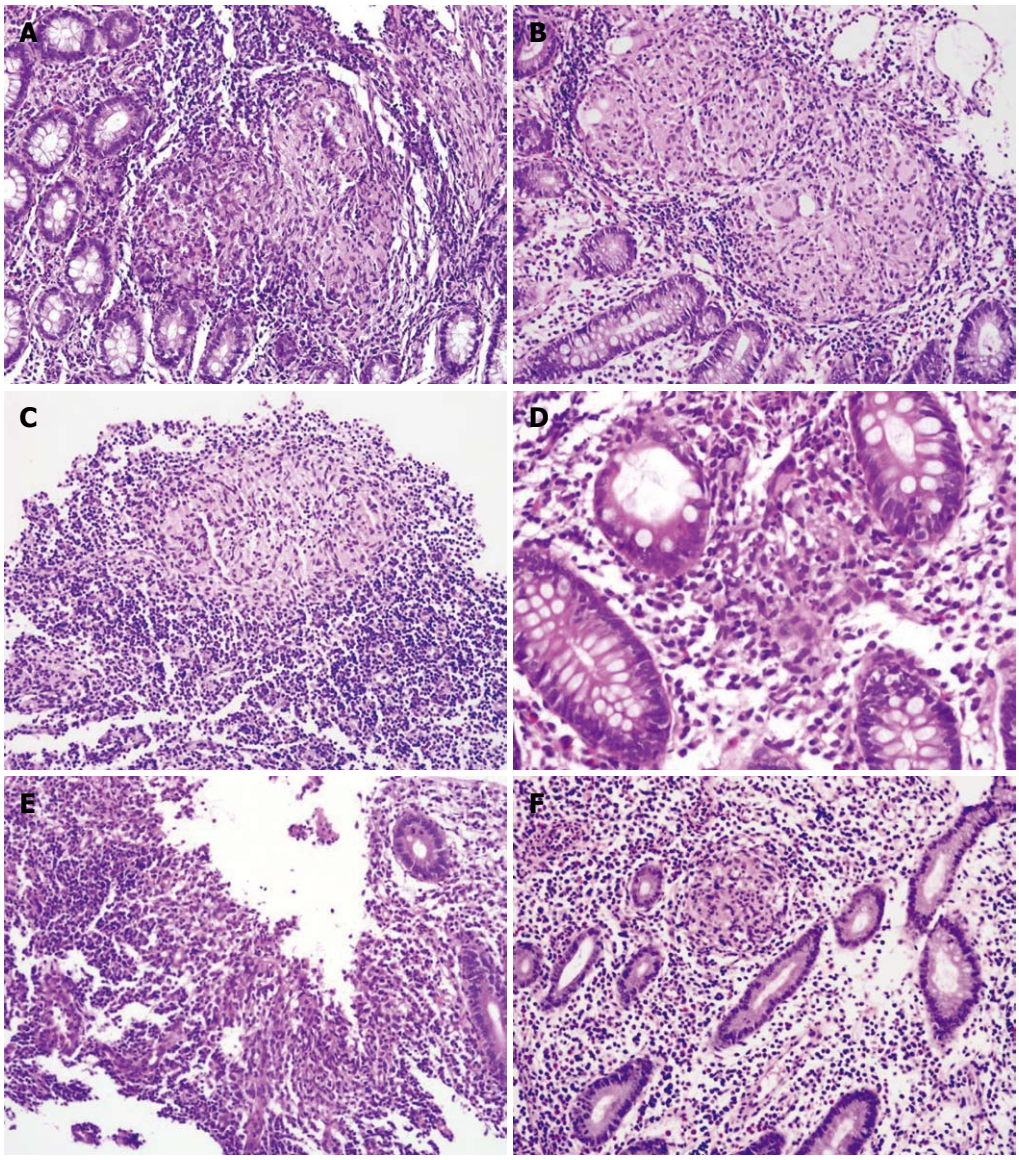


Figure 1 Histopathological parameters of intestinal tuberculosis (ITB) and Crohn's disease (CD). A: Confluent granuloma in ITB; B: Confluent granuloma with caseation necrosis and Langhans giant cells in ITB; C: Granuloma with lymphoid cuff in ITB; D: Vague granuloma in CD; E: Band of epithelioid histiocytes in ulcer base in ITB; F: Small granuloma in CD.

Table 1 Disease location of ITB and CD *n* (%)

Involving site	ITB	CD
Right colon	32 (58.2)	14 (33.3)
Left colon and rectum	4 (7.3)	4 (9.5)
Skip lesion or pancolitis	19 (34.6)	24 (57.1)

ITB: Intestinal tuberculosis; CD: Crohn's disease.

(58.2%), but many of the CD patients showed skip lesion or pancolitis (50.0%) (Table 1).

A summary of the histopathologic features is given in Figure 1 and Table 2. Granulomatous inflammation was seen in 52 cases (94.5%) of ITB and 25 cases (59.5%) of CD. The size and number of granulomas were larger in ITB than in CD ($P = 0.000$). Giant cells ($P = 0.015$), caseation necrosis ($P = 0.003$), confluent granulomas ($P = 0.001$), discrete granulomas ($P = 0.000$), and granuloma with lymphoid cuff ($P = 0.037$) were more frequently associated with ITB. Therefore, these histopathologic features were helpful in differentiating ITB and CD.

When all cases of ITB and CD were divided into four groups according to histopathologic parameters, almost all cases of group Ⅰ and Ⅱ were ITB patients except for one CD patient in group Ⅱ. However, more than half of the ITB patients (52.7%) and nearly all of the CD patients (98%) were in group Ⅲ or group Ⅳ (Table 3). Hence, groups Ⅰ and Ⅱ could be combined to form an "ITB group" although there was one case of CD. Groups Ⅲ and Ⅳ could be combined to form a "confusing group" because differential diagnosis between ITB and CD was difficult based on histopathologic parameters. According to this two-tiered classification, diagnostic sensitivity, specificity, PPV, and NPV were 47.3%, 98%, 96.3%, and 58.6%, respectively.

Table 2 Comparison of histopathologic parameters between ITB and CD *n* (%)

Histologic parameters	Cases		*P* value
	ITB (*n* = 52)	CD (*n* = 26)	
Size of granuloma			0.000
Small (< 200 μm)	6 (11.5)	13 (50.0)	
Medium (200-400 μm)	7 (13.5)	7 (26.9)	
Large (> 400 μm)	39 (75.0)	5 (19.2)	
Granuloma/piece > 1[1]	24 (46.2)	9 (34.6)	0.331
Giant cells	25 (45.5)	5 (19.2)	0.015
Caseation necrosis	14 (26.9)	0 (0.0)	0.003
Confluent granuloma	24 (46.2)	2 (7.8)	0.001
Discrete granuloma	30 (57.7)	3 (11.5)	0.000
Lymphoid cuff	31 (59.6)	9 (34.6)	0.037
Ulcer with histiocytes[2]	17 (30.9)	3 (11.5)	0.056

[1]More than one granuloma per biopsy piece; [2]Ulcer with band of epithelioid histiocytes.

Table 3 Classification of ITB and CD according to histopathologic parameters *n* (%)

Group	ITB (*n* = 55)	CD (*n* = 42)	Re-classification of ITB (*n* = 55)
Ⅰ	14 (25.5)	0 (0.0)	ITB group: 26 (47.3)
Ⅱ	12 (21.8)	1 (2.3)	
Ⅲ	23 (41.8)	15 (35.7)	Confusing group: 29 (52.7)
Ⅳ	6 (10.9)	26 (61.9)	

Group Ⅰ showing caseation necrosis; Group Ⅱ showing more than four kinds of histopathologic parameters without caseation necrosis; Group Ⅲ showing one to four kinds of histopathologic parameters without caseation necrosis; Group Ⅳ without any histopathologic parameters.

TB-PCR in positive and negative control

Among the 9 AFB culture-positive samples, 8 cases were identically TB-PCR positive based on analyses done with kit <A> and kit <B>. However, only 6 cases were TB-PCR positive by the in-house PCR method. Ten normal samples were all negative on three kinds of TB-PCR tests (Table 4).

TB-PCR in ITB and CD

When using kit <A>, 25 samples among 55 ITB cases were PCR positive (sensitivity 45.5%). In contrast, 37 samples among 42 CD cases were PCR negative (specificity 88.1%). When kit <B> was used, 20 samples were PCR positive among the ITB cases (sensitivity 36.4%), and all samples were PCR negative among the CD cases (specificity 100%). Both PCR tests were significantly different between ITB and CD ($P = 0.000$). Figure 2 demonstrates positive bands and negative results of PCR tests using kits <A> and <B>. The sensitivity of the in-house PCR method (5.5%) was much lower than that of either kit (Table 5).

Diagnostic value of TB-PCR in combination with histopathologic parameters

Among the ITB patients, group I showed the highest TB-PCR positivity, which gradually decreased in groups

Table 4 Results of TB-PCR in gold standard

	Positive control (*n* = 9)	Negative control (*n* = 10)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Kit <A>	8	10	88.9	100	100	90.9
Kit <B>	8	10	88.9	100	100	90.9
In-house	6	10	66.7	100	100	71.4

TB-PCR: Polymerase chain reaction for *Mycobacterium tuberculosis*; PPV: Positive predictive value; NPV: Negative predictive value.

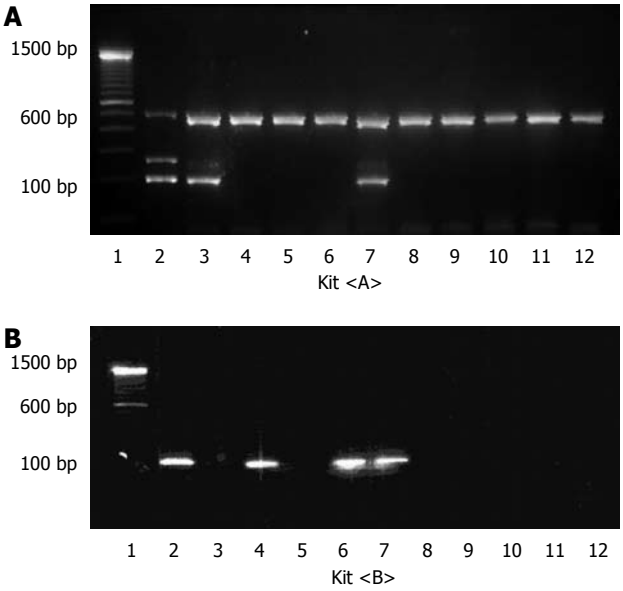


Figure 2 Polymerase chain reaction (PCR) product by kit <A> and kit <B>. A: Number 3 and 7 showing positive result by kit <A>; B: Number 2, 4, 6, 7 showing positive result by kit <B>.

Ⅱ, Ⅲ, and Ⅳ (Table 6). On two-tiered classification, the ITB cases belonging to the "ITB group" showed 57.7% (kit <A>) and 53.8% (kit <B>) TB-PCR positivity. In the "confusing group" there were 10 additional PCR-positive cases among the ITB cases based on kit <A> analysis, and 6 additional PCR-positive cases based on ITB analysis by kit <B>. The PCR positive cases in the "confusing group" of ITB could be reclassified as the "ITB group". The one CD case classified as group Ⅱ by histopathologic evaluation revealed negative TB-PCR. After a combination of histopathologic and PCR analyses with kit <B>, the diagnostic sensitivity of ITB was significantly increased ($P = 0.010$) to 65.5% (Table 7). Although the sensitivity of kit <A> was better than that of kit <B>, the diagnostic usefulness was not validated due to low specifity. The diagnostic sensitivity and specificity of histopathologic findings and adjuvant effect of TB-PCR is summarized in Table 7.

DISCUSSION

A number of reports have documented the characteristics of ITB[15-17] and CD[18-20], and some have also compared the histopathological features. Pulimood *et al*[8,9]

Table 5 Results of TB-PCR in ITB and CD

TB-PCR test	ITB (n = 55)		CD (n = 42)		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	P value
	+	-	+	-					
Kit <A>	25	30	5	37	45.5	88.1	83.3	55.2	0.000
Kit <B>	20	35	0	42	36.4	100	100	54.5	0.000
In-house	3	52	0	42	5.5	100	100	44.7	0.256

Table 6 Histopathologic parameters and TB-PCR n (%)

Confirmed diagnosis	Group of HPP	n	TB-PCR positive cases		
			Kit <A>	Kit <B>	In-house
ITB	Ⅰ	14 (25.5)	9 (64.3)	10 (71.4)	2 (14.3)
	Ⅱ	12 (21.8)	6 (50.0)	4 (33.3)	0 (0.0)
	Ⅲ	23 (41.8)	9 (34.6)	6 (23.1)	1 (3.8)
	Ⅳ	6 (10.9)	1 (33.3)	0 (0.0)	0 (0.0)
CD	Ⅰ	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Ⅱ	1 (2.3)	0 (0.0)	0 (0.0)	0 (0.0)
	Ⅲ	15 (35.7)	4 (15.4)	0 (0.0)	0 (0.0)
	Ⅳ	26 (61.9)	1 (6.3)	0 (0.0)	0 (0.0)

HPP: Histopathologic parameters.

Table 7 Comparison of diagnostic accuracy of ITB before and after combination of histopathology and TB-PCR

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	P value
Histopathology	47.3	100	100	59.2	
Combination with kit <A>	65.5	88.1	87.8	66.1	0.390
Combination with kit <B>	58.2	100	100	64.6	0.010
Combination with in-house PCR	49.1	100	100	60.0	0.320

evaluated several histological parameters in colonoscopic biopsies to distinguish between ITB and CD. They examined characteristics of granuloma such as size, number, confluence, caseation necrosis, location, and presence of microgranulomas, as well as ulceration pattern, focally enhanced colitis, and disruption of submucosa. They reported that all of these characteristics were significantly different between ITB and CD, but other factors, such as architectural alteration, chronic inflammation, and discontinuous inflammation, were not significantly different. In our study, the histopathological features that had been reported as favorable factors for ITB in the literature were also statistically significant in differentiating theses two diseases. More than a half of ITB cases (52.7%) showed less than five kinds of these histopathologic parameters confused with CD microscopically. Therefore, differential diagnosis of ITB and CD based on histopathologic features may be limited.

PCR detection of mycobacteria in histopathological specimens has been introduced as a rapid and useful technique for the diagnosis of pulmonary and extrapulmonary tuberculosis[13,21,22]. *IS6110* was originally described in 1990 as a 1.36 kb insertion sequence with extensive numerical and positional polymorphism, found only in the *M. tuberculosis* complex (MTBC). Many members of the MTBC contain multiple *IS6110* copies[23]. Because there are multiple copies of the *IS6110* insertion sequence in the MTBC, *IS6110* is an attractive target for PCR amplification. However, some MTBC strains do not contain the *IS6110* sequence[24], so false negative results may occur. Past studies have shown that detection of the *IS6110* gene in *M. tuberculosis* has variable sensitivity (30%-90%)[11,25-28] and specificity (60%-100%)[12,13,21,29].

MPB64 is a mycobacterial antigen specific to the *M. tuberculosis* complex. This antigen is secreted from *M. tuberculosis*, *Mycobacterium bovis* (*M. bovis*), and some strains of *M. bovis* BCG during bacterial growth[30,31]. In a study of pleural and meningeal tuberculosis, the sensitivity and specificity of nested-PCR using *MPB64* were 70% and 88%[32]. The study of Singh *et al*[33] used two PCRs targeting *IS6110* and *MPB64* and found that sensitivity is 77.8% better than PCR targeting *IS6110* only (48.2%).

The current study used conventional in-house PCR, which detects the *IS6110* gene sequence, along with two newly developed kits manufactured by <A> and <B>. Kit <A> detects the *MPB64* and *IS6110* genes simultaneously using the duplex nested PCR method, and kit <B> detects the *IS6110* gene sequence using only the nested PCR method. Among 9 positive control cases, 8 cases (88.9%) were PCR positive for both kits. The sensitivity of the in-house PCR test (66.7%) was lower than that of both kits. Ten negative control cases were all negative for three kinds of PCR test. The kits we used showed higher sensitivity and specificity than in-house PCR. The false negative rates of the kits were lower than in-house PCR test.

The PCR test performed on the endoscopic biopsy specimens showed a dramatic drop in sensitivity in both kits, as well as in the in-house method compared to the control samples. False negative results were probably due to paucibacilli. All samples were obtained from endoscopic biopsies, so the tissue samples were small in amount. *M. tuberculosis* would not be distributed evenly in the tissue, furthermore, each case tested for PCR was only a 50 μm thick sample from each paraffin block of endoscopic biopsy. Hence, some sections among the ITB samples might not have contained *M. tuberculosis*. The absence or only a few copies of *IS6110* gene sequence in a proportion of *M. tuberculosis*[34,35] may also bring about a false negative result.

The sensitivity of kit <A> was better than that of kit <B>. Twenty-five cases of ITB (45.5%) were posi-

tive by kit <A>, but 20 cases (36.4%) were positive by kit <B>. This suggests that the combined method could reduce false negativity in the samples without the *IS6110* sequence, as Singh *et al*[33] reported. However, five cases of CD were TB-PCR positive by kit <A>. Therefore, the specificity of kit <A> was not as good as that of kit <B> (100%). The false-positive results on the duplex method might be due to contamination or the influence of gene sequences on each other during the experiments.

The histopathologic findings were very informative in differentiating ITB and CD. However, more than half of all ITB cases (52.7%) and nearly all CD cases fell in the "confusing group". So, in order to increase diagnostic accuracy, it is better to interpret histopathologic features in conjunction with PCR results. Because of the high specificity of TB-PCR, we could ascertain ITB if the TB-PCR test was positive. The TB-PCR test could detect *M. tuberculosis* in 20.7%-34.5% of these gray zone ITB cases, and the diagnostic sensitivity of ITB was significantly increased ($P = 0.010$) from 47.3% to 58.2% with kit <B>, but the low sensitivity of the test in small biopsy pieces was problematic. Sufficient tissue sampling might improve results. Also, it is necessary to check the clinical features and endoscopic findings first.

In conclusion, kit <A> and kit <B> TB-PCR were more sensitive in detecting *M. tuberculosis* in endoscopic biopsy specimens when compared to the in-house PCR method. The TB-PCR test with kit <B> targeting *IS6100* combined with histopathologic examination appears to be a helpful technique in differentiating ITB and CD in endoscopic biopsy samples.

COMMENTS

Background

The incidence of intestinal tuberculosis (ITB) is increasing with resurgence of tuberculosis (TB). Because of similar clinical, endoscopic, radiological, and microscopic findings the differential diagnosis of ITB with Crohn's diseases (CD) is very difficult. Similar histopathological features of chronic colitis are seen in both ITB and CD but with some delicate differences. Recent studies have shown that polymerase chain reaction for *Mycobacterium tuberculosis* (TB-PCR) is useful for diagnosis of TB.

Research frontiers

Some characteristics of individual granulomas and some other histopathological features might be helpful for differential diagnosis between ITB and CD and TB-PCR targeting *IS6100* and/or *MPB64* is sensitive and specific for diagnosis of TB. But which and how many kinds of characteristic histopathological features may be helpful for differential diagnosis and whether combination with TB-PCR targeting *IS6100* and/or *MPB64* in endoscopic biopsy specimen may improve differential diagnosis are not well known.

Innovations and breakthroughs

In this study, the authors found that caseation necrosis and more than four kinds of histopathological features in an endoscopic biopsy specimen are very informative in differential diagnosis of ITB and CD and these histopathological features combined with TB-PCR can improve the differential diagnosis. Otherwise the commercial kits are more sensitive and specific in TB-PCR in endoscopic biopsy specimen than in-house TB-PCR.

Applications

By selecting and observing endoscopic biopsy specimens from patients of ITB and CD and comparing TB-PCR using commercial kits and in-house TB-PCR, this study is beneficial for the differential diagnosis between ITB and CD practically, and provides initial insight into a method of diagnosis of TB.

Peer review

This paper is very nicely written and makes a good contribution.

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ORIGINAL ARTICLE

Clinicopathological features and prognosis assessment of extranodal follicular dendritic cell sarcoma

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Abstract

AIM: To establish a model for prognosis assessment of extranodal follicular dendritic cell (FDC) sarcoma.

METHODS: Nine lesions were examined by routine and molecular approaches. Clinicopathological factors from the new cases and 97 reported cases were analyzed for their prognostic values.

RESULTS: The current lesions were found in five male and four female patients, located mainly in the head and neck area and averaging 7.2 cm in size. Six patients had recurrence or metastasis and three remained free of disease. The 106 patients (male/female ratio, 1.1:1) were aged from 9 to 82 years (median, 44 years). The tumor sizes ranged from 1.5 to 21 cm (mean, 7.4 cm). Abdominal/pelvic region was affected most frequently (43%). Surgical resection was performed in 100 patients, followed by radiation and/or chemotherapy in 35 of them. Follow-up data were available in 91 cases, covering a period of 3-324 mo (mean, 27 mo; median, 19 mo). Of the informative cases, 38 (42%) had recurrence or metastasis, and 12 (13%) died of the disease. These tumors were classified histologically into low- and high-grade lesions. A size $\geqslant$ 5 cm (P = 0.003), high-grade histology (P = 0.046) and a mitotic count $\geqslant$ 5/10 HPF (P = 0.013) were associated with tumor recurrence. The lesions were defined as low-, intermediate- and high-risk tumors, and their recurrence rates were 16%, 46% and 73%, and their mortality rates 0%, 4% and 45%, respectively.

CONCLUSION: Extranodal FDC tumors behave like soft tissue sarcomas. Their clinical outcomes are variable and can be evaluated according to their sizes and grades.

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Key words: Extranodal follicular dendritic cell sarcoma; Prognosis assessment; Histologic grade; Immunohistochemistry; *In situ* hybridization; Mutation detection

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INTRODUCTION

Follicular dendritic cell (FDC) sarcoma was described by Monda *et al*[1] in 1986. It is considered to be derived from FDC, whose cell type normally forms a tight meshwork in the primary and secondary lymphoid follicles and participates in the immune system by interacting with B or T lymphocytes[2]. Epstein-Barr virus (EBV) infection was demonstrated in majority of the hepatic and splenic lesions and its causative effect has been proposed for the pathogenesis of the lesion[3-9], but the association is not evident in most of the tumors from other sites[10-13]. Majority of the tumors were found in lymph nodes. However, about one-third of the lesions were identified in extranodal sites. It was considered that extranodal FDC sarcoma occurred preferentially in the head and neck area[14].

FDC sarcoma is usually regarded as an indolent tumor with a tendency of local recurrence but a low risk of metastasis, behaving like a low-grade soft tissue sarcoma[2,15]. However, Perez-Ordonez *et al*[16] considered this tumor more aggressive through their observation of 13 cases. Because of the rarity of the tumor, assessment of its prognosis remains difficult, though intra-abdominal location, a high mitotic count (≥ 5/10 HPF), coagulative necrosis and marked cellular atypia were proposed to be predictors of an unfavorable outcome[3]. With more cases encountered in our pathological practice and reported from literatures, various morphologic and clinical representations were noticed. Clearly, histological grade of this malignancy remains to be defined and a model is needed to evaluate the recurrence risk of individual tumors within this category.

In this study, we presented our experience in nine cases of FDC sarcoma from northern China. In addition, clinicopathological features and corresponding data extracted from 97 reported cases were analyzed, with special respect to prognosis assessment of this disease.

MATERIALS AND METHODS

Samples and histological examination

As listed in Table 1, the tissue specimens were resected from nine patients in three medical centers, including the Fourth Military Medical University Tangdu Hospital in Xi'an (Cases 1 and 2), Chinese Academy of Medical Sciences Cancer Hospital in Beijing (Cases 3-6, 8 and 9) and the First Affiliated Hospital of Inner Mongolia Medical College in Huhehot (Case 7). These patients were admitted for a solid occupation in head and neck areas (n = 6), and thoracic and abdominal cavities (n = 3) from 2002 to 2009. Cases 1 and 2 had been described in a brief report[17], and follow-up data were complemented in the current study.

The formalin-fixed, paraffin-embedded tissue blocks were retrieved from the files. Sections of 4 μm in thickness were prepared and stained with hematoxylin and eosin (HE). Three pathologists (Su Q, Yang HY and Li L) reviewed the slides independently, and the diagnosis was confirmed by their histological and immunohistochemical phenotypes. As described previously[2], whorl, storiform and fascicular arrangements of spindle tumor cells were regarded as architectural features for typical lesions, while epithelioid and pleomorphic patterns were considered anaplastic phenotypes in this study. Other morphologic parameters, including mitotic activity, coagulative necrosis, nuclear atypia, distribution of infiltrating small lymphocytes and tumor sites, were also assessed. In addition, we also evaluated proportions of the typical tumor components, as described above, in each lesion.

Immunohistochemistry

Immunohistochemical reactions were performed on paraffin sections following deparaffinization, rehydration and antigen retrieval. The antigen retrieval was performed by heating in citrate (pH 6.0) or ethylene-diamine-tetraacetic acid (EDTA) buffer (pH 9.0; Table 2). In addition to routine markers for tumor diagnosis, such as pan-cytokeratins (CKs; AE1/AE3), epithelial membranous antigen (EMA), vimentin, desmin and smooth muscle-type actin (SMA), a panel of antibodies were applied to demonstrate histiocytic and dendritic cell linage differentiation, including those against CD68, S100 protein, CD21, CD23, CD35, CD1a and podoplanin (D2-40). Ki-67 antigen was detected to show proliferative activity of each lesion, and p53 protein accumulation was demonstrated by reaction with the antibody DO-7. After incubation with primary antibodies at room temperature for 1 h, the antigen-antibody reaction was demonstrated by a horseradish peroxidase (HRP)-labeled secondary antibody (EnVision™ Detection System, Dako A/S, Glostrup, Denmark) at room temperature for 15 min and visualized in a solution containing 0.5 mg/mL 3,3'-diaminobenzidine (DAB) and 0.01% hydrogen peroxide. Finally, the sections were counterstained slightly with hematoxylin and mounted in resin.

The expression levels were evaluated semiquantitatively according to the reaction intensities and percentages of immunoreactive cells, and expressed as strong (3+), moderate (2+), weak (+) and negative (-). Ki-67 antigen expression levels were assessed based on the number of positive cells/1000 nucleated tumor cells from randomly selected 5 high-power fields, and expressed as Ki-67 antigen-labeling indices (Ki-67-LI) in percentages. The p53 protein expression levels were evaluated by percentages of positive cells as described previously[18] and expressed as high (3+, > 30%), moderate (2+, 5%-30%), low (1+, < 5%) and absent (-, 0%).

In situ hybridization

In situ hybridization was performed in seven cases to demonstrate EBV-encoded RNA (EBER) molecules using a detection kit (Triplex International Biosciences Co. Ltd., Fuzhou, China) according to instructions of the manufacturer. Briefly, sections were treated with proteinase K following deparaffinization and rehydration. After denaturation for 90 min at 55℃, the sections were incubated with a digoxin-labeled DNA probe overnight at 37℃, and washed with phosphate-buffered saline. The hybridization signals were demonstrated by consecutive reactions with

Table 1 Clinicopathological and therapeutic data of nine new cases of extranodal FDC sarcoma (Cases 1-9)

Case No.	Age (yr)	Gender	Sites	Size (cm)	HG	MC (/10 HPF)	Year of onset	Initial diagnoses	Initial treatment	Recurrence, DFT (mo)	Disease status	Follow-up (mo)
1	60	M	Tonsil	5.0	Low	< 1	2002	Granuloma	Surg + RT		NED	86
2	35	F	Parapharyn-geal space	5.0	High	10	2003	NPC	Surg	LR, 2	DOD	12
3	63	M	Infratem-poral fossa	4.0	Low	1	2003	PNET	Surg + RT + ChT		NED	72
4	30	F	Pyform sinus	5.0	High	9	2004	FDC sarcoma	Surg + RT	DM (lung), 25	DOD	25
5	23	M	Mediastinum	8.0	Low	3	2006	MPNST	Surg + RT + ChT	DM (bone), 45	AWD	45
6	45	M	Liver	14.5	Low	< 1	2007	FDC sarcoma	Surg		NED	27
7	36	F	Mesentery	15.0	High	7	2007	Malignant GIST	Surg	DM (liver, ovary), 4	AWD	27
8	28	F	Parapharyn-geal space	6.0	High	3	2008	FDC sarcoma	Surg + RT + ChT	DM (lung), 14	AWD	22
9	55	M	Tonsil	2.0	High	9	2008	FDC sarcoma	Surg + RT	LR, 18	AWD	21

AWD: Alive with disease; ChT: Chemotherapy; DFT: Disease-free time following operation; DM: Distant metastasis; DOD: Died of disease; GIST: Gastrointestinal stromal tumor; HG: Histological grades; LR: Local recurrence; MC: Mitotic counts; MPNST: Malignant nerve sheath tumor; NED: No evidence of disease; NPC: Nasopharyngeal carcinoma; PNET: Primitive neuroepidermal tumor; RT: Radiotherapy; Surg: Surgery; FDC: Follicular dendritic cell.

Table 2 Primary antibodies and antigen retrieving procedures

Antibodies	Origins and clones	Dilutions	Antigen retrieval	Sources
Pan-cytokeratin	MAb, AE1/AE3	1:80	MW-citrate	Invitrogen
EMA	MAb, Mc5	1:100	MW-citrate	Invitrogen
Vimentin	MAb, V9	1:200	MW-citrate	NeoMarker
Desmin	MAb, ZC18	1:80	MW-citrate	Invitrogen
SMA	MAb, IA4	1:100	Not treated	Invitrogen
CD45	MAb, RP2/18	1:120	MW-citrate	Novacastro
CD68	MAb, KP1	1:200	MW-citrate	Zeta
S-100 protein	PAb, rabbit	1:400	MW-citrate	Dako
CD21	MAb, 2G9	1:20	AC-EDTA	NeoMarker
CD23	MAb, SP23	1:25	MW-citrate	NeoMarker
CD35	MAb, KuN241	1:20	AC-EDTA	NeoMarker
CD1a	MAb, MTB1	1:20	MW-citrate	Zeta
Podoplanin	MAb, D2-40	1:50	AC-EDTA	Zeta
p53 protein	MAb, DO-7	1:100	AC-EDTA	NeoMarker
Ki-67 antigen	MAb, MIB-1	1:200	AC-EDTA	Zymed

Antibody suppliers: Dako, Glostrup, Denmark; Invitrogen Corporation, Carlsbad, CA, USA; NeoMarkers Inc., Fremont, CA, USA; Novacastro Laboratories Ltd., Newcastle upon Tyne, UK; Zeta Corporation, Sierra Madre, CA; Zymed Laboratories, San Diego, CA, USA. EMA: Epithelial membrane antigen; MAb: Mouse monoclonal antibody; PAb: Polyclonal antibody; SMA: Smooth muscle-type actin; MW-citrate: Microwaving for 8 min in citrate buffer (pH 6.0); AC-EDTA: Autoclaving for 100 s in EDTA buffer (pH 9.0).

a mouse monoclonal antibody against digoxin and the polymerized HRP-labeled anti-mouse immunoglobulin G, and visualized by incubation in a solution containing DAB and hydrogen peroxidase. Finally, the slides were counterstained slightly with hematoxylin and mounted with resin. A case of Hodgkin lymphoma, known to be positive for EBER, was used as a positive control. Yellow or brown staining of tumor cell nuclei was considered positive.

Amplification and sequencing of P53 gene

As most of the documented mutations of *P53* gene occur in its exons 5-8[19,20], these regions were examined for mutations by nested (for exons 6 and 7) or semi-nested polymerase-chain reaction (PCR; for exons 5 and 8). The primers used were adopted from literatures[21,22]. Their sequences were as follows: exon5s, 5'-TCTGTCTCCTTCCTCTTCCTA-3'; exon5as, 5'-AACCAGCCCTGTCGTCTCT-3'; exon6s, 5'-TTGCTCTTAGGTCTGGCCCC-3'; exon6as, 5'-CAGACCTCAGGCGGCTCATA-3'; exon7s, 5'-TTGCTCTTAGGTCTGGCCCC-3'; exon7as, 5'-GGGTCAGCGGCAAGCAGAGG-3'; exon8s, 5'-GACAAGGGTGGTTGGGAGTAGATG-3'; exon8as, 5'-GCAAGGAAAGGTGATAAAAGTGAA-3'. Selected tumor tissues were collected from serial paraffin sections of 10 μm in thickness. Following deparaffinization and rehydration, the tissues were digested by incubation with proteinase K (20 mg/mL) at 56℃ for 72 h. Genomic DNA was extracted with a kit (QIAamp DNA Mini Kit, Qiagen GmbH, Hiden, Germany) following instructions of the manufacturer. Primer pairs used for the first-round amplification of exons 5, 6, 7 and 8 were exon5s/exon6as, exon5s/exon7as, exon6s/exon8as and exon7s/exon8as, respectively. The reactions were in a mixture of 10 μL containing 100 ng of DNA templates, 0.8 μL of deoxynucleotide triphosphate (2.5 mmol/L each), 1 μL of 1 μmol/L sense and antisense primers each, 1 μL 10 × buffer (100 mmol/L Tris-HCl, pH 8.3, containing 500 mmol/L KCl and 20 mmol/L $MgCl_2$) and 0.5 U of *Taq* DNA polymerase (Takara Biotechnology Co., Ltd., Dalian, China). Amplification was conducted for 35 cycles (94℃, 40 s; 55℃, 40 s; 72℃, 40 s for exons 5 and 7; 1 min for exons 6 and 8) following the initial denaturation at 94℃ for 5 min. The final elongation was at 72℃ for 10 min. The second-round PCR was performed in a mixture of 50 μL containing 3 μL of products of the first-round reaction and 1 μL of 5 μmol/L sense and antisense inner premiers each. Primer pairs used for amplifying exons 5, 6, 7 and 8 were exon5s/5as, exon6s/6sa, exon7s/7as and exon8s/8as, respectively. The cycling conditions were the same as the first-round reaction. Amplification efficiency and specificity were visualized by electrophoresis on an agarose gel (2%), and the products of exons 5, 6, 7 and 8 migrated at positions of 196, 81, 234 and 277 bp, respectively. The products were subjected to direct sequencing using an automatic system (Prism 3100 Genetic Analyzer, Applied Biosystems, Foster, CA, USA) as described previously[22,23]. The sequences obtained were compared with those from Genbank (www.gdb.org).

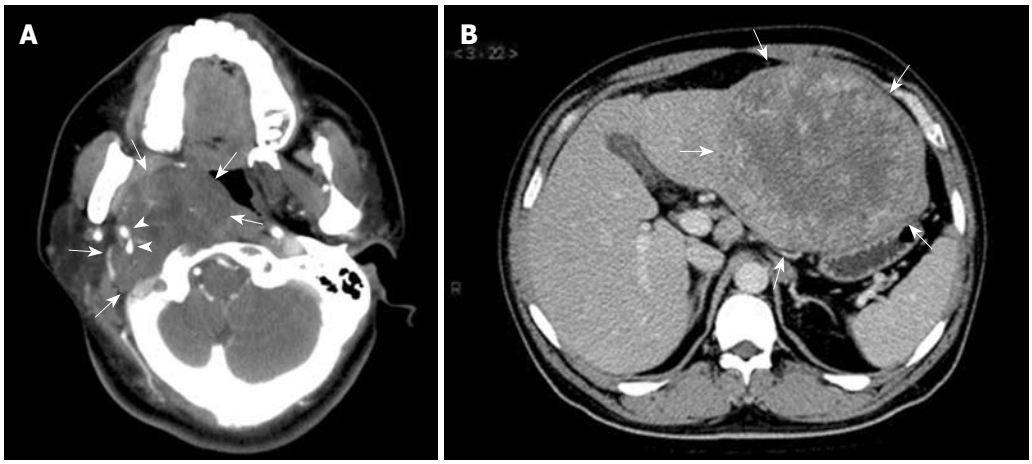


Figure 1 Radiological features of extranodal follicular dendritic cell (FDC) sarcoma shown by computed tomographic scan. A: Case 8, a tumor (arrows) at the right parapharyngeal space showing soft tissue-like density and an expansive growth pattern, with the internal and external carotid arteries (arrowheads) engulfed; B: Case 6, a well-circumscribed mass (arrows) at the left lobe of liver, showing irregular enhancement at its periphery.

Literature review

Search of literatures was performed using MEDLINE on PubMed (www.ncbi.nlm.nih.gov/pubmed) with the terms "follicular dendritic cell tumor" or "follicular dendritic cell sarcoma" combined with "extranodal". Articles published in Chinese journals were found by searches on Wanfangdata (www.wanfangdata.com.cn). References of the collected articles were also reviewed to identify other relevant publications. Efforts were made to identify cases that were reported more than once in different settings and only one entry with the most updated information was included for such cases. Nodal tumors were not included in this study.

Statistical analysis

Statistical analyses were carried out using the Software Packages for Social Science 13.0 for Windows (SPSS, Inc, Chicago, IL, USA). Survival of patients was assessed using the data obtained from both the current study and the literatures. Overall and recurrence-free survival rates were calculated and analyzed using the Kaplan-Meier method. Associations of different pathological factors with clinical outcomes were described by the Chi-square test. Fisher's exact test was also used when necessary. *P* values below 0.05 were considered significant.

RESULTS

Current cases

Ages of the nine patients (five men and four women) ranged from 23 to 63 years (median, 36 years). A local mass was the primary complaint in eight cases (Figure 1), and a sore throat was the first symptom in one patient (Case 2). Sizes of the tumors ranged from 2.0 to 15.0 cm as measured by the longitudinal dimension, averaging 7.2 cm. A diagnosis of FDC sarcoma was made in four of the nine cases following pathologic examination of the resected specimens. For Cases 1, 2, 3, 5 and 7, the resected lesions were misdiagnosed as granulomatous inflammation suspicious of tuberculosis, nasopharyngeal carcinoma (NPC), primitive neuroectodermal tumor (PNET), malignant peripheral nerve sheath tumor (MPNST) and malignant gastrointestinal stromal tumor (GIST), respectively. The diagnosis of FDC sarcoma was established by re-examination and immunohistochemistry with the delayed periods ranging from one to 66 mo. Their clinicopathological features are listed in Table 1. No precursor lesions were recorded in any of them.

Table 3 Histological features of low- and high-grade FDC sarcomas

Parameters	Low-grade phenotypes	High-grade phenotypes
Architectures	Nodular patterns in whorl, storiform and fascicular arrangement	Diffuse and sheet-like patterns
Cellular features	Spindle or blunt cells with mild nuclear atypia and a small nucleolus in > 90% of area	Epithelioid or pleomorphic cells with marked nuclear atypia and large nucleoli
Mitotic counts	< 5/10 HPF	⩾ 5/10 HPF
Ki-67-LI	< 10%	⩾ 10%
Necrosis	Absent	Frequently present
Lymphocyte infiltration	Sporadic and throughout the lesion	Focal or regional

Typically, the lesions were composed of spindle and ovoid tumor cells arranged in whorl, fascicular and storiform patterns, showing mild nuclear atypia and sparkled with small lymphocytes (Figure 2). Of the nine tumors, three (Cases 1, 5 and 6) were composed uniformly of the typical components, and one (Case 3) showed typical components in > 90% of the area and epithelioid appearance in small foci (Figure 3A). In the remaining five cases, large areas of epithelioid (Cases 2 and 8) and pleomorphic tumor cell components (Cases 4, 7-9), arranged in a diffuse or sheet-like pattern and with moderate to marked nuclear atypia, were also identified (Figure 3B-D). Sporadic small lymphocytes were present throughout tumor tissues in all the four typical cases (Figure 2), and this feature was less prominent (Figure 3B) or even absent (Figure 3C and D) in the rest five lesions. Focal coagulative necrosis was identified in six cases (Cases 2, 3, 5-8). The hepatic lesion (Case 6)

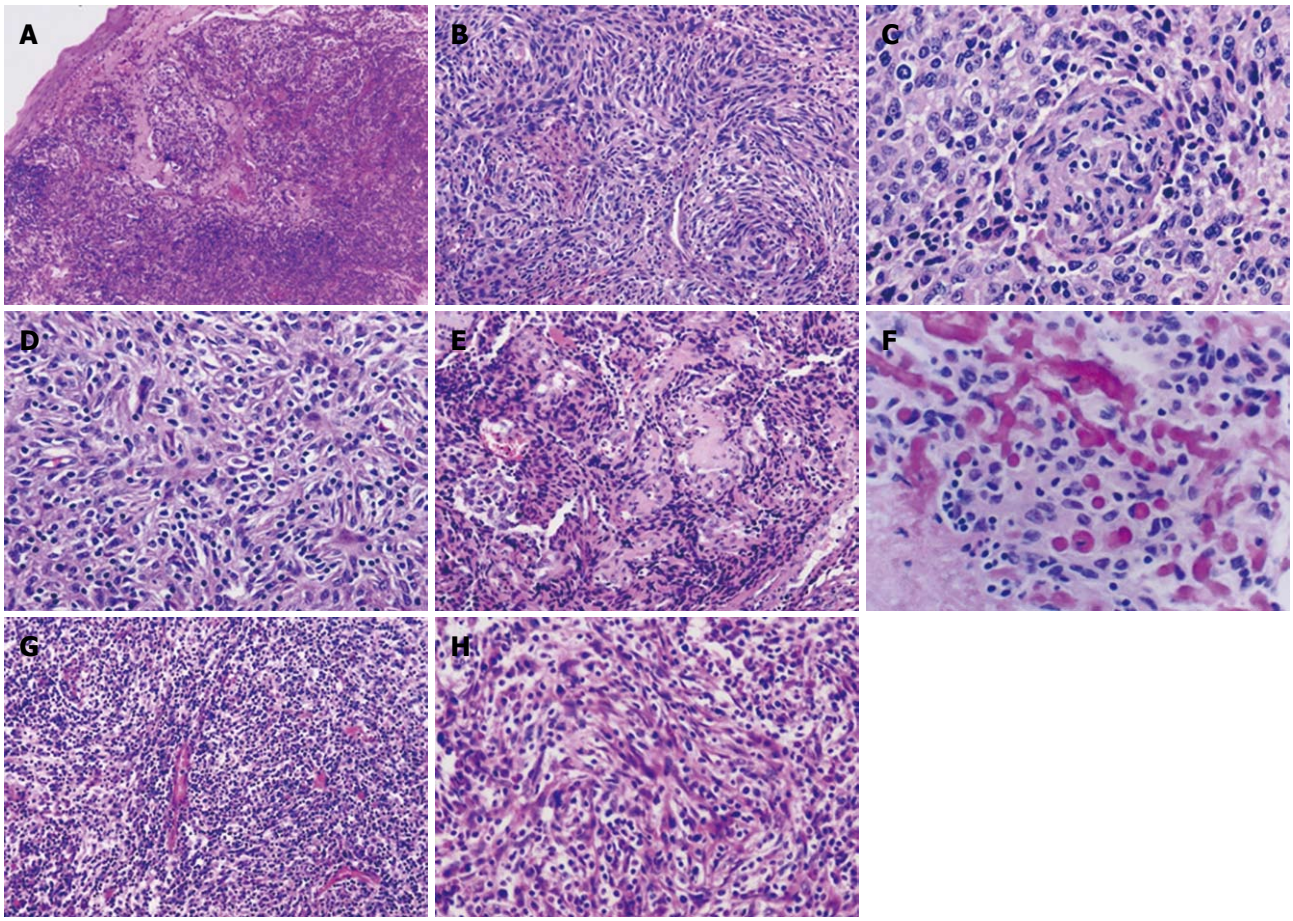


Figure 2 Typical features of follicular dendritic cell sarcoma of the conventional (A-F) and inflammatory pseudotumor-like types (G and H). Spindle and ovoid tumor cells, frequently growing in nodules as in Case 1 (A), arrange in whorl (Case 3, B and C) and storiform (Case 3, D) and fascicular patterns (Case 6, H), with sprinkling small lymphocytes throughout the former type of lesions and numerous plasma cells and lymphocytes in the latter. Perivascular sclerosis was noted in Case 3 (E), with foci of osteoid matrix deposition resembling osteosarcoma (F). HE: A, × 40; B, E and G, × 100; C, D, F and H, × 200.

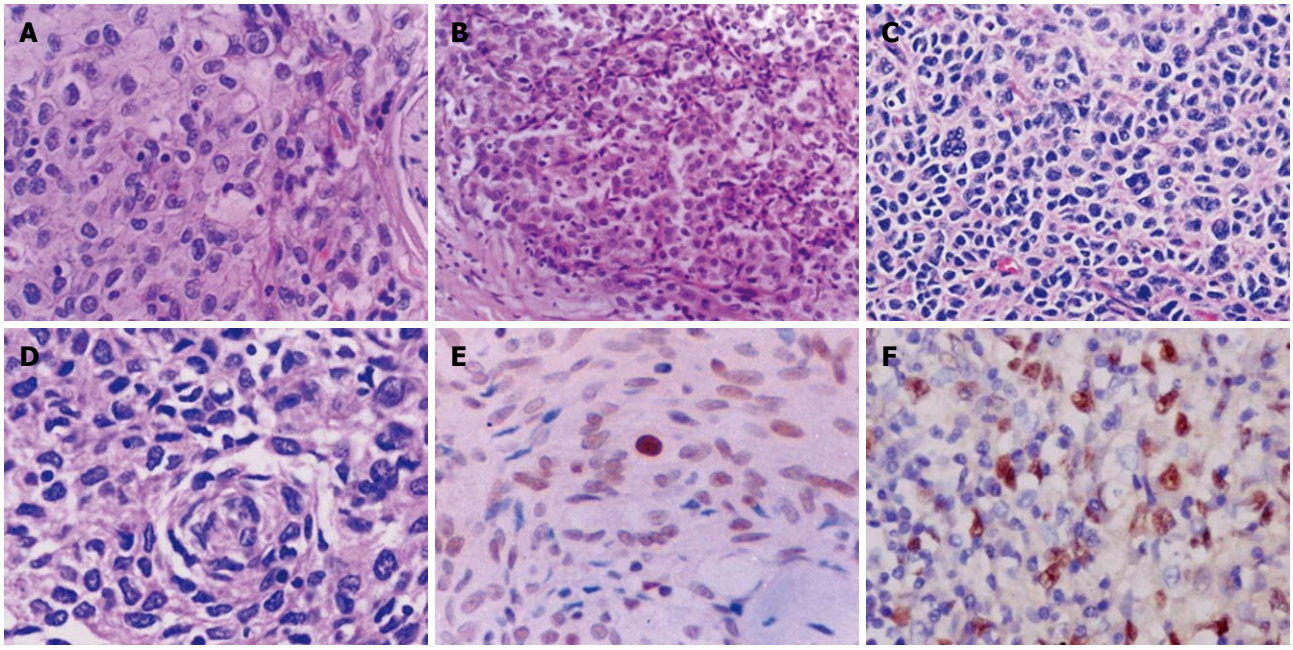


Figure 3 Atypical morphology of FDC sarcoma (A-D) and expression of p53 protein (E) and Epstein-Barr virus-encoded RNA (EBER) (F) in tumor cells. A-D: Epithelioid (A and B) and pleomorphic tumor cells (C and D) are arranged in a sheet-like or diffuse pattern. Lymphocyte infiltration is less prominent (A and B) or absent (C and D) in these areas. HE: A, C and D, × 400; B, × 200; E: Nuclear immunoreactivity for p53 protein in majority of tumor cells. S-P, × 400; F: *In situ* hybridization signal for EBER in tumor cells, × 400.

Table 4 Expression of lineage-specific and kinetic-associated molecules and EBV-encoded RNA (EBER) in nine FDC sarcomas (Cases 1-9)

Cases	CK	EMA	Vimentin	CD45	CD21	CD23	CD35	Podoplanin	CD68	S-100 protein	p53 protein[1]	Ki-67-LI (%)	EBER
1	-	1+	3+	-	3+	NT	1+	NT	NT	NT	3+	4	NT
2	-	-	NT	-	2+	NT	2+	NT	NT	-	2+	15	NT
3	-	1+	2+	-	2+	3+	-	3+	NT	-	2+	1	-
4	-	-	3+	-	3+	2+	2+	-	-	1+	2+	8	-
5	-	-	3+	-	2+	2+	3+	2+	1+	2+	2+	15	-
6	-	-	NT	-	1+	2+	-	-	1+	1+	-	10	+
7	-	-	2+	-	1+	3+	3+	-	-	-	2+	40	-
8	-	-	1+	-	3+	2+	-	2+	2+	1+	2+	20	-
9	-	-	2+	-	1+	2+	1+	-	-	-	2+	35	-

[1]Evaluated by the percentages of positive tumor cells, although majority of the positive cells showing weak nuclear immunoreactivity. Immunoreactivities expressed as strong (3+), moderate (2+), weak (1+) and negative (-); NT: Not tested.

was composed of spindle cells with mild nuclear atypia and numerous inflammatory cells including plasma cells, resembling an inflammatory pseudotumor (Figure 2G and H). In addition, neutrophils infiltration was observed in two lesions (Cases 3 and 8). In some tumor areas (Case 3), vascular proliferation was prominent with perivascular sclerosis (Figure 2E) and focal deposition of osteoid matrix mimicking osteosarcoma (Figure 2F).

As described above, the histology varies greatly among the lesions. It is conceivable to predict clinical outcomes using pathological parameters. According to our own data and proposals by other authors[3], we defined the criteria for low- and high-grade FDC sarcomas using six parameters. As shown in Table 3, the former four were found to be decisive and regarded as major factors for the grading. The lesions with typical architectural and cellular phenotypes, mitotic counts below 5/10 HPF and Ki-67-LI below 10%, as in Cases 1, 3, 5 and 6, were classified as low-grade tumors, and those with anaplastic morphology, mitotic counts up to 5/10 HPF and/or Ki-67-LI up to 10%, as in Cases 2, 4, 7-9, as high-grade tumors. The rest two parameters, including necrosis and loss or reduction of infiltrating lymphocytes, were found to be useful adjuvant factors, but not prerequisites for establishing a diagnosis of the high-grade lesion.

As listed in Table 4, all of the nine lesions were positive for vimentin and negative for CK and CD45. Weak immunoreactivity for EMA was observed in two cases. A diagnosis of FDC sarcoma was established by its immunohistochemical phenotypes including positivity for CD21 (9/9, 100%), CD23 (7/7, 100%) and CD35 (6/9, 67%). Podoplanin was detected in three (43%) of the seven cases. CD68 and S100 protein were detected in about half of the lesions, but their expression levels were low to moderate. Nuclear accumulation of p53 protein, albeit at relatively low levels, was observed in most of the tumor cells in eight (89%) of the nine lesions (Figure 3E). Amplification and sequencing were successful for *P53* exons 5-8 in one lesion (Case 9), and we failed to show any mutation. Ki-67-LI in these lesions was quite variable, ranging from 1% to 40%. *In situ* hybridization was performed in seven of the lesions for EBER, with positive signals demonstrated only in the hepatic lesion (Case 6, Figure 3F).

As the initial treatment, surgery was performed for all of the lesions, with the tumor masses completely removed in seven cases. In Cases 1 and 8, complete resection was not fulfilled because of the involvement of the internal carotid artery. Adjuvant treatment was given to six of the patients (Cases 1, 3-5, 8 and 9) after the operation. Radiation was administered in all the cases and chemotherapy in three cases (Cases 3, 5 and 8). The remaining three patients (Cases 2, 6 and 7) rejected any adjuvant treatment. Follow-up was carried out in all the cases for a period of 12-86 mo, with a median of 27 mo. As shown in Table 1, seven (78%) patients remained alive, four of them had recurrence or metastasis and three were alive without event, and two (22%) died of the tumor recurrence. Of the six cases with events, two recurred locally and four had distant metastases, with a disease-free period below 3 years.

Clinicopathologic features of extranodal FDC sarcomas

Data of 106 extranodal FDC sarcomas, including nine new cases (Table 1) and 97 from literatures (Table 5), were extracted and reviewed carefully. Of the patients, 56 were men and 50 were women, with a ratio of 1.1:1. Ages of the patients ranged from 9 to 82 years at diagnosis, with their mean being 46 years and median being 44 years. The tumors were identified at different anatomic regions. The abdominal and pelvic region was affected in 46 cases (43%), including 14 in the liver, 13 in abdominal/pelvic soft tissues, five in the spleen, and 14 at other organs including pancreas (n = 3), stomach (n = 3), colon and rectum (n = 3), appendix (n = 2), ampulla of Vater (n = 1), small intestine (n = 1) and adrenal (n = 1). Of the five splenic cases, two also showed liver involvement. These hepatic lesions were regarded as metastasis in this study. Head and neck were another common region for FDC sarcoma (38/106, 36%), with tonsils and parapharyngeal space affected most frequently. Fourteen (13%) of the lesions were identified from thoracic cavity, of which nine were located at mediastinum, three at lung, one at pleura and one at the chest wall. The less frequent sites included breast (n = 3), soft tissues at thigh (n = 2), dura mater (n = 1), groin (n = 1) and skin (n = 1). Tumor sizes ranged from 1.5 to 21 cm as described in longitudinal dimensions, with a mean of 7.4 cm. A broad spectrum of histological

Table 5 Clinicopathologic and therapeutic data of 97 cases of extranodal FDC sarcoma obtained from literatures (Cases 10-106)

Cases No.	Origins	Age (yr)	Gender	Size (cm)	Sites	Initial treatment (responses)	Recurrence, DFT (mo)	Status	Follow-up (mo)
10	Chan *et al*[24], 1994	44	M	1.5	Tonsil	Surg		NED	36
11	Chan *et al*[24], 1994	63	F	3	Palate	Surg + RT		NED	54
12	Hollowood *et al*[25], 1995	23	F	6.5	Small intestine	Surg	LR (2 times), 6	AWD	24
13	Hollowood *et al*[25], 1995	63	M	NA	Pancreas head	Surg	LR, 8	DOD	11
14	Nayler *et al*[26], 1996	18	F	2	Tonsil	Surg + ChT		NA	NA
15	Perez-Ordonez *et al*[16], 1996	40	F	NA	Abdomen	Surg + ChT	LR and DM (liver), 12	AWD	24
16	Perez-Ordonez *et al*[16], 1996	62	F	NA	Tonsil	Surg		NED	12
17	Perez-Ordonez *et al*[16], 1996	62	M	NA	Mediastinum	Surg + ChT	DM (lung), 24	AWD	24
18	Perez-Ordonez *et al*[16], 1996	46	M	NA	Mediastinum	Surg + RT		NED	12
19	Perez-Ordonez *et al*[16], 1996	31	M	NA	Mediastinum	Surg		NED	10
20	Perez-Ordonez *et al*[16], 1996	75	F	NA	Spleen	Surg	LR, 11	DOD	11
21	Selves *et al*[27], 1996	68	F	NA	Liver	Surg + ChT		NED	30
22	Shek *et al*[28], 1996	35	F	NA	Liver	Surg	LR (2 times), 30	AWD	60
23	Chan *et al*[3], 1997	42	M	8	Mesocolon, involving LN and peritoneum	Surg + ChT	LR, 18	DOD	18
24	Chan *et al*[3], 1997	32	M	NA	Tonsil	Surg + RT	LR and DM (LN), 54	AWD	54
25	Chan *et al*[3], 1997	40	F	7	Parapharyngeal space	Surg	LR, 12	AWD	12
26	Chan *et al*[3], 1997	17	M	11	Neck soft tissue	Surg + ChT		NA	NA
27	Chan *et al*[3], 1997	25	F	7	Neck soft tissue	Surg + RT	DM (lung), 20	AWD	20
28	Beham-Schmid *et al*[29], 1998	44	M	2	Nasopharynx	Surg + RT + ChT		NED	20
29	Shek *et al*[30], 1998	37	M	1.5	Liver	Surg		NED	24
30	Shek *et al*[30], 1998	61	F	4	Ampulla of Vater	Surg		NED	9
31	Araújo *et al*[31], 1999	14	M	1.5	Hard palate	Surg		NED	5
32	Desai *et al*[32], 1999	45	F	NA	Parapharyngeal space	Surg	LR, 31	NED	57
33	Fisher *et al*[33], 1999	41	F	2.5	Breast	Surg	LR and DM (LN), 36	NED	36
34	Galati *et al*[34], 1999	65	F	1.5	Thyroid	Surg + RT		NED	36
35	Schwarz *et al*[35], 1999	62	M	15	Abdominal wall	Surg + RT		NED	8
36	Choi *et al*[36], 2000	28	F	4	Neck soft tissue	Surg	DM (lung), 324	AWD	324
37	Choi *et al*[36], 2000	66	F	NA	Neck soft tissue	Surg + RT	DM (lung), 24	AWD	24
38	Han *et al*[37], 2000	45	M	5	Stomach	Surg		NED	10
39	Chan *et al*[38], 2001	34	M	NA	Nasopharynx	Surg		NED	36
40	Chang *et al*[39], 2001	37	F	5	Ascending colon	Surg		NED	7
41	Chen *et al*[5], 2001	57	F	9.5	Liver	Surveillance		AWD	36
42	Chen *et al*[5], 2001	51	F	15	Liver	Surg		NED	12
43	Chiaramonte *et al*[40], 2001	39	M	NA	Retroperitoneum	Surg	DM (LN and lung), 6	DOD	6
44	Shah *et al*[41], 2001	33	M	9.5	Lung	Surg + ChT		NA	NA
45	Biddle *et al*[11], 2002	33	M	NA	Pharynx	Surg + RT	DM (lung), 10	AWD	10
46	Biddle *et al*[11], 2002	48	F	1.5	Tonsil	Surg		NED	6
47	Biddle *et al*[11], 2002	48	M	3.5	Tonsil	Surg		NED	8
48	Pileri *et al*[2], 2002	42	M	NA	Mediastinum	Surg		NED	NA
49	Vargas *et al*[42], 2002	54	F	3	Tonsil	Surg		NED	8
50	Vargas *et al*[42], 2002	54	F	6	Left parotid	Surg + RT	LR, 6	AWD	8
51	Ceresoli *et al*[43], 2003	35	M	NA	Mediastinum	ChT + RT (Prog)		DOD	7
52	Satoh *et al*[44], 2003	16	M	3	Tonsil	Surg + RT + ChT		NED	24
53	Geerts *et al*[45], 2004	40	F	NA	Stomach, with DM (liver)	Surg		AWD	5
54	Grogg *et al*[46], 2004	62	M	NA	Left-thigh soft tissue	Surg		NED	18
55	Grogg *et al*[46], 2004	57	F	NA	Tonsil, involving LN	Surveillance		AWD	8
56	Grogg *et al*[46], 2004	64	F	14	Spleen, with DM (liver)	Surg + ChT		NED	5
57	Kröber *et al*[47], 2004	76	M	NA	Mediastinum	Surg + RT		NED	24
58	O'Malley[48], 2004	38	M	5	Cecum	Surg		NA	NA
59	Shi *et al*[49], 2004	37	M	1.5	Tonsil	Surg + ChT		NED	36
60	Bradshaw *et al*[50], 2005	9	M	5	Neck soft tissue	Surg	DM (lung), 60	NED	96
61	Kazakov *et al*[13], 2005	38	M	1.8	Skin	Surg		NA	NA
62	Khalid *et al*[51], 2005	19	F	16	Liver	ChT (PR)		DOD	24
63	Torres *et al*[52], 2005	82	M	15	Liver	Surg		NED	18
64	Aydin *et al*[53], 2006	76	F	3.5	Tonsil	Surg + RT		NED	48
65	Choi *et al*[54], 2006	68	M	NA	Dura mater	Surg + RT		NED	7
66	Clement *et al*[55], 2006	27	F	4	Tonsil	Surg + RT		NED	6
67	Díaz de Liaño *et al*[56], 2006	NA	NA	NA	Abdominal cavity	Surg	DM (liver), 18	NED	18
68	Gan *et al*[57], 2006	32	F	4.5	Parotid	Surg		NED	20
69	Jiang *et al*[58], 2006	46	F	8.4	Anterior mediastinum	ChT (stable)	DM (bone), 12	NED	19
70	Jiang *et al*[59], 2006	46	F	21	Pelvic/abdominal cavity, multiple	Surg		NED	6
71	Kovács *et al*[60], 2006	65	M	4	Lung	Surg		NED	18
72	Li *et al*[61], 2006	19	M	NA	Nasal cavity	Surg		NED	11
73	Lu *et al*[62], 2006	72	F	7	Groin	Surg		NA	NA
74	Shen *et al*[63], 2006	64	M	10.5	Pancreas	Surg	DM (liver), 18	NED	24

75	Shia *et al*[64], 2006	30	M	NA	Rectum	Surg	LR, 10	AWD	15
76	Shia *et al*[64], 2006	29	F	12	Lesser omentum	Surg		NED	17
77	Shia *et al*[64], 2006	69	F	NA	Tonsil	Surg + RT	DM (hilar LN+lung), 96	AWD	108
78	Xu *et al*[65], 2006	16	M	5	Adrenal	Surg		NA	NA
79	Chang *et al*[66], 2007	64	M	16	Chest wall	Surg		NED	15
80	Leipsic *et al*[67], 2007	43	M	13	Mediastinum	Surg		NA	NA
81	Padilla-Rodríguez *et al*[68], 2007	35	M	21	Retroperitoneal space	Surg + RT		NED	24
82	Sander *et al*[69], 2007	44	F	11	Spleen	Surg	DM (thorax, liver and kidney), 4	DOD	9
83	Soriano *et al*[70], 2007	25	F	6	Pelvis	Surg	LR, 2, 2nd Surg+RT	NED	14
84	Soriano *et al*[70], 2007	56	M	2	Pancreas, involving LN	Surg + RT	LR, 2	AWD	7
85	Soriano *et al*[70], 2007	33	M	NA	Nasopharynx	Surg + RT	LR, 10	DOD	14
86	Soriano *et al*[70], 2007	64	F	4	Spleen, with liver involved	Surg + ChT		AWD	29
87	Soriano *et al*[70], 2007	66	M	16	Pleura	ChT (Prog)		DOD	7
88	Tu *et al*[12], 2007	63	M	15	Jejunum mesentery	Surg		NA	NA
89	Tu *et al*[12], 2007	43	M	4	Appendix	Surg		NA	NA
90	Tu *et al*[12], 2007	28	F	15	Stomach	Surg	DM (liver), 3	AWD	3
91	Yuan *et al*[71], 2007	29	M	10	Liver	Surg	LR, 6	AWD	6
92	De Pas *et al*[72], 2008	40	F	4	Breast	Surg		NED	62
93	De Pas *et al*[72], 2008	53	F	NA	Liver	Surg	DM (LN), 11	DOD	22
94	De Pas *et al*[72], 2008	64	F	2.1	Breast	Surg		NED	20
95	Granados *et al*[73], 2008	57	F	13	Liver	Surg		NED	24
96	Liu *et al*[74], 2008	42	M	12	Gastrocolic omentum	Surg		NA	NA
97	Peng *et al*[75], 2008	60	M	7	Mesentery	Surg		NA	NA
98	Zhang *et al*[76], 2008	36	M	NA	Nasopharynx	Surg		NED	5
99	Zhang *et al*[76], 2008	32	F	NA	Spleen	Surg		NA	NA
100	An *et al*[77], 2009	40	M	5	Liver	Surg		NED	3
101	Denning *et al*[78], 2009	64	F	1.7	Lung	Surg		NED	24
102	Liu *et al*[79], 2009	75	M	4	Liver	Surg		NA	NA
103	Romero-Guadarrama *et al*[80], 2009	54	F	15	Thigh soft tissue	Surg	LR, 12	NED	48
104	Shen *et al*[81], 2009	43	M	5	Appendix	Surg	LR, 8	NED	8
105	Vaideeswar *et al*[82], 2009	50	M	2.5	Tonsil	Surg		NED	48
106	Xu *et al*[83], 2009	57	F	11	Liver	Surg		NA	NA

LN: Lymph node; NA: Not available; Prog: Disease progression.

phenotypes were described, while the most helpful diagnostic features were the typical arrangement of spindle and ovoid tumor cells with eosinophilic cytoplasm, indistinct cytoplasmic borders and syncytial appearance, and presence of small lymphocytes in the tumor.

Morphologic information, indicative of the architectural and cellular anaplasia, was collected from the articles and re-evaluated carefully. Of the 63 informative cases, 37 (59%) were described as lesions with mild atypia, and 26 (41%) with moderate to severe cytological atypia throughout or in a certain area of the tumor. In the current study, these two groups were classified into low-grade and high-grade lesions, respectively. All of the tumors occurring in the liver resembled an inflammatory pseudotumor in morphology. Five of the eight informative cases were divided into low-grade group, and the other three into high-grade group due to identification of anaplastic components. Coagulative necrosis was recorded in 29 (45%) of the 64 informative cases. Mitotic counts were provided in 61 cases, with 31 (51%) up to 5/10 HPF. Ki-67-LI was calculated in 20 lesions, with 14 (70%) up to 10%. Preoperative tumor spreading was identified in 15 (23%) of the 66 informative cases through pathological examination of the resected samples, including local lymph node involvement in 11, distant metastasis to liver or peritoneal dissemination in three, and both nodal involvement and peritoneal spreading in one.

Management and clinical outcomes

One hundred and four patients received therapeutic procedures and two rejected any treatment. Of the patients, 100 (94%) were treated surgically to remove the tumor. Adjuvant treatment was administered in 35 cases, including radiation in 20, chemotherapy in 10 and both procedures in five. Chemotherapy was performed in most of the cases based on CHOP regimen including cyclophosphamide, adriamycin, vincristine and prednisone.

Follow-up data were available in 91 cases. The observation periods ranged from 3 to 324 mo, with a mean and a median of 27 and 19 mo, respectively. Overall, 38 patients (42%) had local recurrence and/or distant metastasis during the adjuvant treatment or surveillance period, with the events occurring within 36 mo in most (33/37, 89%) of the cases. Local recurrence occurred in 21 patients (23%) at a median of 12 mo after surgical removal (range, 3-31 mo). Distant metastases occurred in 19 patients (21%) at a median of 14 mo after operation (range, 4-324 mo). Metastatic sites included lung (n = 9), liver (n = 6), lymph node (n = 5), bone (n = 1), thorax (n = 1), ovary (n = 1) and kidney (n = 1). At the last follow-up, 12 patients (13%) died of the tumor, 25 (27%) were alive with disease, and 54 (59%) were alive with no evidence of disease. The 2-year and 5-year overall survival rates for the entire group were 82% and 79%, and their 2-year and 5-year disease-free survival rates were 57% and 32%, respectively (Figure 4).

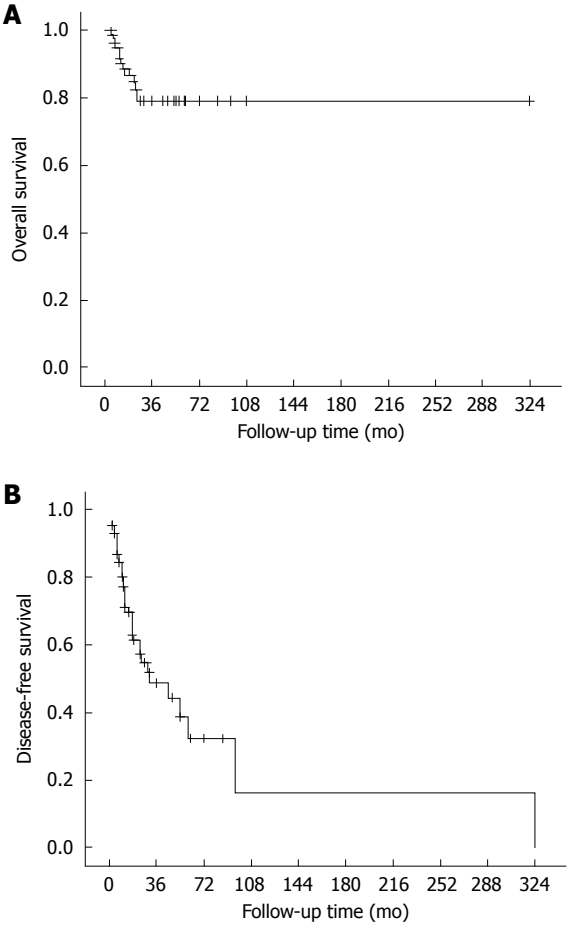


Figure 4 Overall (A) and disease-free survival curves (B) of FDC sarcoma. The results are based on follow-up data of 91 informative cases.

Prognostic factors

Associations between nine clinicopathological factors and recurrence risk were studied, and the results are summarized in Table 6. Of the 60 informative lesions, 25 (42%) were smaller than 5 cm and 34 (58%) were up to 5 cm in their longitudinal dimensions. Four (16%) of the smaller lesions relapsed (two with local recurrence and two with distant metastasis) and 19 (56%) of the larger lesions relapsed (nine with local recurrence and 10 with metastasis). The recurrence rate was markedly elevated with the increase of tumor size ($P = 0.003$). In addition, a mortality rate of 17% was observed for cases with the larger lesions, and none of the patients with smaller lesions died of the disease ($P = 0.036$).

For most of the lesions, as listed in Table 5, histological grades were assessed mainly according to descriptions on architectural features and cellular atypia from respective literatures. For this reason, tumor necrosis, mitotic activity and Ki-67-LI were evaluated separately in this study. Of the 63 informative cases, 37 (59%) were evaluated as low-grade and 26 (41%) as high-grade lesions. Twelve (32%) of the low-grade lesions relapsed (seven with local recurrence, four with metastasis and one with both local recurrence and metastasis) and 15 (58%) of the high-grade lesions relapsed (seven with local recurrence, seven with metastasis and one with both local recurrence and metastasis). The recurrence rate was higher in the latter group than in the former ($P = 0.046$). In addition, the high-grade lesions resulted in death in nine (35%) of the cases, while only one (3%) of the patients with low-grade lesions died of the disease, the mortality rate being closely associated with tumor grade ($P = 0.001$). Eighteen (58%) of the 31 lesions with a mitotic count ≥ 5/10 HPF relapsed, the frequency being higher than those with a count < 5/10 HPF (8/30, 27%, $P = 0.013$). The mortality rate (7/31, 23%) was also higher in the former group than in the latter (0/30, $P = 0.011$). According to the limited number of informative cases, increased Ki-67-LI (≥ 10%) appeared to indicate an unfavorable clinical outcome, but its impact failed to reach statistical significance ($P = 0.628$). The patients younger than 50 years tended to develop tumor recurrence more frequently (51%) than those over 50 years of age (31%), but without statistically significant difference ($P = 0.072$). Other factors, including gender, necrosis and therapeutic procedures following surgery, were not related to clinical outcomes ($P > 0.05$).

Table 6 Associations between clinicopathological parameters and recurrence risk in patients with extranodal FDC sarcoma

Pathologic parameters	Recurrence frequencies (%)	*P* values
Age (yr)		
< 50	26/51 (51)	0.072
≥ 50	11/35 (31)	
Gender		
Male	15/40 (38)	0.290
Female	22/46 (48)	
Size (cm)		
< 5	4/25 (16)	0.003
≥ 5	19/34 (56)	
Histological grades		
Low	12/37 (32)	0.046
High	15/26 (58)	
Mitotic counts		
< 5/10 HPF	8/30 (27)	0.013
≥ 5/10 HPF	18/31 (58)	
Ki-67-LI		
< 10%	2/6 (25)	0.628
≥ 10%	8/14 (57)	
Necrosis		
Absent	17/35 (49)	0.307
Present	13/29 (45)	
Treatments		
Surgery alone	22/52 (42)	0.590
Surgery + adjuvant therapy	15/31 (48)	
Sites		
Abdominal/pelvic	18/37 (49)	0.372
Other sites	20/53 (38)	

The associations of tumor size, histological grade and mitoactivity with overall and disease-free survival are described in Figure 5. Among them, tumor size and histological grade were the most important factors, respectively, for tumor recurrence (Figure 5B) and disease-associated death (Figure 5C). Thus, a model was established for the recurrence risk assessment by combining the former two parameters. Recurrence rates of lesions smaller than 5 cm, the larger lesions (≥ 5 cm) with low-grade histology and those with high-grade histology were 16% (4/25), 46%

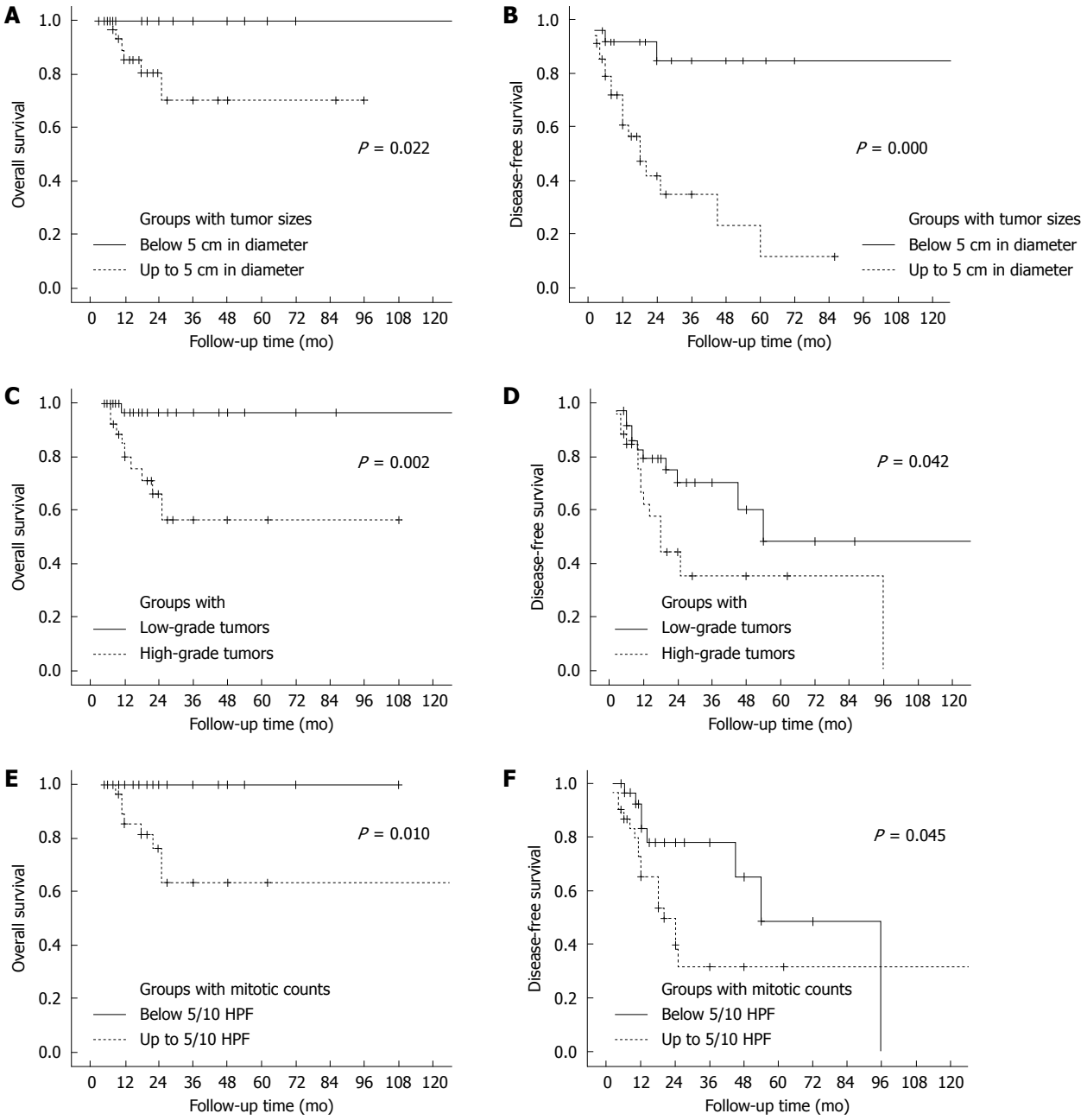


Figure 5 Overall (A, C and E) and disease-free survival curves (B, D and F) of patients with FDC sarcoma, showing data of 120 mo.

(11/24) and 73% (8/11), respectively, their difference being significant ($P = 0.000$). Based on the recurrence potential, these three groups of lesions were designated as low-, intermediate- and high-risk FDC sarcomas, respectively (Figure 6). A markedly higher mortality rate was also observed in cases with a high-risk tumor (45%) than in those with the low-risk (0%, $P = 0.001$) and intermediate-risk lesions (4%, $P = 0.006$). The low-risk group had a longer survival and the high-risk group had the most unfavorable clinical outcomes considering both the tumor recurrence and mortality (Figure 7).

During the follow-up period, 18 (49%) of the 37 informative lesions from abdominal and pelvic cavities recurred (20/53, 38%, $P = 0.302$). Of the 37 abdominal/pelvic lesions, 12 occurred in the liver and 25 were identified from other tissues. A lower recurrence risk (3/12, 25%) was observed in hepatic lesions than in the extrahepatic lesions (15/25, 60%, $P = 0.046$). A more favorable disease-free survival was also observed for the former group ($P = 0.004$). However, there was no significant difference between the hepatic (2/12, 17%) and extrahepatic groups (5/25, 20%) in mortality ($P = 0.372$, Figure 8). The size of the hepatic lesions (mean, 9.5 cm; range, 2-21 cm) was comparable to that of the extrahepatic lesions (mean, 10.4 cm; range, 1.5-16 cm, $P = 0.644$). Morphologically, all of the hepatic lesions belonged to the inflammatory pseudotumor-like variant, and most of the extrahepatic lesions were classified into conventional type. However, we failed to show a significant difference in frequency of occurrence of high-

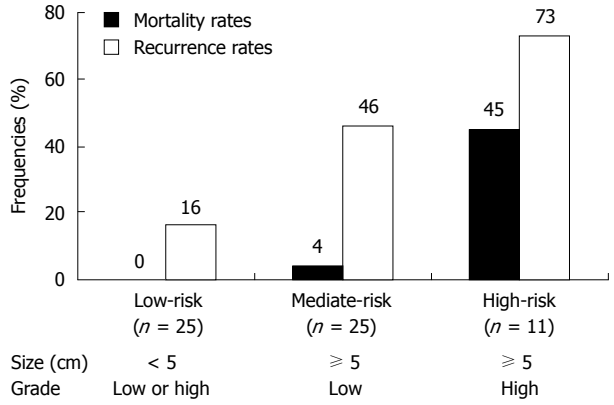


Figure 6 Definitions of low- (low-risk), intermediate- (mediate-risk) and high-risk groups (high-risk) of extranodal FDC sarcomas. Mortality rates: overall, $P = 0.000$; low-risk *vs* mediate-risk, $P = 1.000$; low-risk *vs* high-risk, $P = 0.001$; mediate-risk *vs* high-risk, $P = 0.006$; Recurrence rates: overall, $P = 0.001$; low-risk *vs* mediate-risk, $P = 0.032$; low-risk *vs* high-risk, $P = 0.002$; mediate-risk *vs* high-risk, $P = 0.167$.

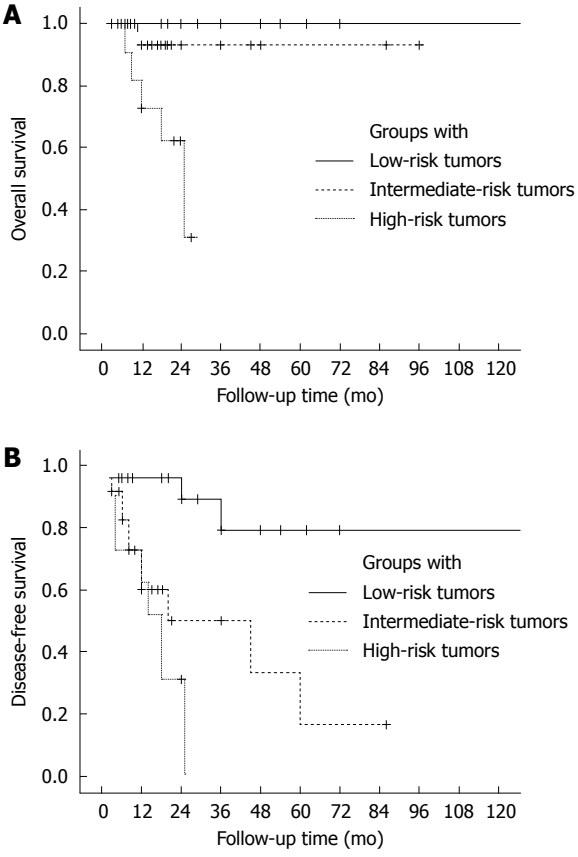


Figure 7 Overall (A) and disease-free survival curves (B) of patients with FDC sarcoma, estimated in groups with low-, intermediate- and high-risk tumors, showing data of 120 mo. A: Overall, $P = 0.000$; low-risk *vs* mediate-risk, $P = 0.273$; low-risk *vs* high-risk, $P = 0.000$; mediate-risk *vs* high-risk, $P = 0.017$; B: Overall, $P = 0.000$; low-risk *vs* mediate-risk, $P = 0.003$; low-risk *vs* high-risk, $P = 0.000$; mediate-risk *vs* high-risk, $P = 0.207$.

grade component between the hepatic (3/8, 38%) and extrahepatic lesions (7/18, 39%, $P = 1.000$).

The extrahepatic, abdominal/pelvic lesions were also compared with those occurring out of abdominal/pelvic cavity. Their recurrence rates were 60% (15/25) and 38%

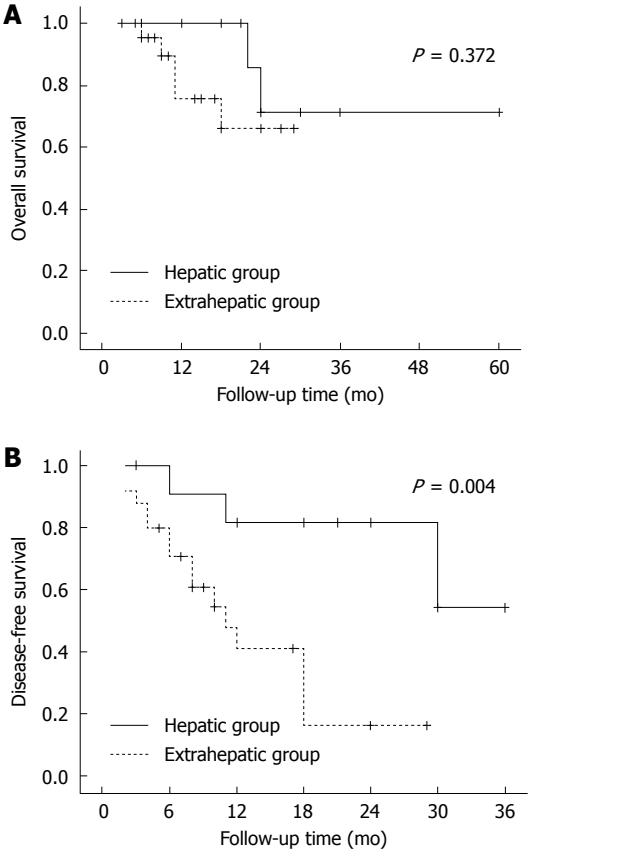


Figure 8 Overall (A) and disease-free survival curves (B) of abdominal/pelvic FDC sarcomas. The lesions are divided into hepatic and extrahepatic groups.

(20/53), respectively, with the former lesions recurring more frequently ($P = 0.000$) and earlier (Figure 9). The former cases tended to have a higher mortality rate (5/25, 20%) than the latter group (5/53, 6%), but without statistically significant difference ($P = 0.051$, Figure 9). These two groups also showed difference in their sizes, with the average of the former group (9.5 cm) being larger than that of the latter group (5.3 cm, $P = 0.003$). They were not significantly different in histological grade ($P = 0.204$), with high-grade phenotypes observed in 57% (26/46) of the former lesions and 39% (7/18) of the latter lesions.

DISCUSSION

FDC sarcoma may occur in extranodal tissues from a variety of anatomical sites, including head and neck, liver, spleen, gastrointestinal tract, soft tissue, skin, lung and breast[14]. As shown in this survey, abdominal/pelvic cavity and head/neck areas are affected more frequently, where 43% and 36% of extranodal FDC sarcomas were identified. It usually presents with a solitary tumor, typically composed of spindle and ovoid cells with scattered mature lymphocytes. However, there is a broad spectrum of morphologic variations in this tumor, some of which overlap with those of other neoplasms including ectopic meningioma, thymoma, myoepithelial tumor, inflammatory myofibroblastic tumor and GIST. In our series, osteoid component was found in a low-grade FDC sarcoma

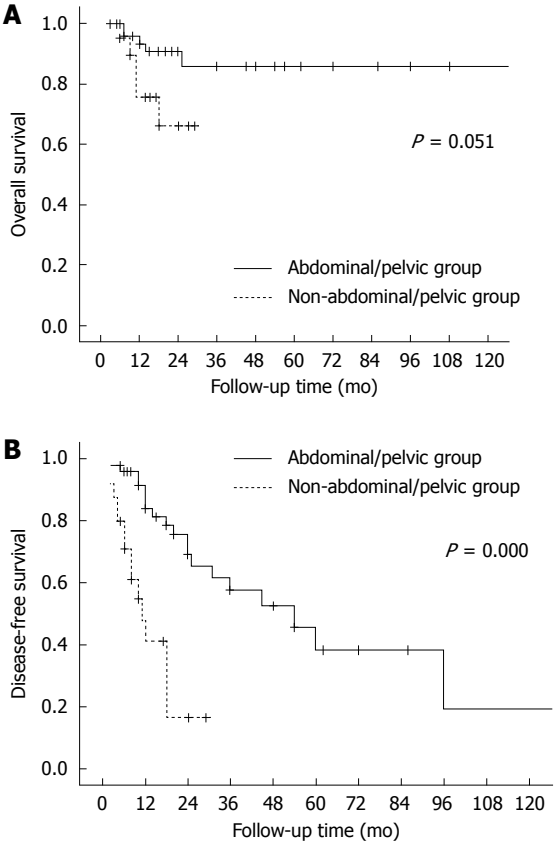


Figure 9 Overall (A) and disease-free survival curves (B) of patients with extrahepatic FDC sarcomas, showing data of 120 mo. The lesions are divided into abdominal/pelvic and non-abdominal/pelvic groups.

(Case 3). This is the first case showing presence of an osteosarcoma-like phenotype (Figure 2F). Misdiagnosis may occur when a pathologist is not aware of the tumor, as in Cases 1 and 2 of the current series (Table 1). The diagnosis can be established by immunohistochemical demonstration of CD21, CD23, CD35[70], podoplanin and clusterin expression[64,84,85].

FDC sarcoma is usually regarded as an indolent tumor with a tendency of local recurrence but a low risk of metastasis, behaving like a low-grade soft tissue sarcoma[15]. In a study of 13 cases, Perez-Ordonez *et al*[16] observed a substantial risk of metastases, prompting consideration of this malignancy to be of intermediate grade. An analysis by Shia *et al*[64] found that the 2- and 5-year recurrence-free survival rates were 62% and 27%, respectively. In our study, a total number of 106 extranodal cases were analyzed. The recurrence rate was 42%, including local recurrence in 23% and distant metastasis in 21% of the cases, and the mortality rates were 13% over the periods from 3 to 324 mo (mean, 27 mo; median, 19 mo). The 2-year and 5-year disease-free survival rates were 57% and 32%, and the 2-year and 5-year overall survival were 82% and 79%, respectively. It appears that extranodal FDC sarcomas, as a whole, are more aggressive than a low-grade soft tissue sarcoma.

As demonstrated in this study, pathologic representations of extranodal FDC sarcoma are variable among different cases. New approaches are needed to find most important prognostic factors relevant to their clinical outcomes. It has been proposed that intra-abdominal involvement, a high mitotic count (≥ 5/10 HPF), coagulative necrosis and marked cellular atypia are potentially helpful predictors of an unfavorable outcome[3]. In this study, nine clinicopathological parameters were analyzed, and large tumor size (≥ 5 cm in diameter), high-grade histology and high mitotic counts (≥ 5/10 HPF) were found to be closely associated with the clinical outcomes. The recurrence risk seemed to be increased in lesions with high Ki-67-LI, but the association showed no statistical significance. It needs to be clarified in future studies with a larger number of informative cases.

While some histological phenotypes, including cellular atypia, mitoactivity and coagulative tumor necrosis, were considered important parameters for prognosis assessment[3], a grading model remains to be established. Soriano *et al*[70] identified five high-grade lesions from 14 FDC sarcomas based on cellular atypia, but they failed to show its relevance of statistical significance to clinical outcomes. In this study, we examined nine new cases from northern China, extracted and reviewed data of 97 cases from literatures, and established histological criteria for low-grade and high-grade FDC sarcomas according to four major parameters including architectural alteration, cellular atypia, mitoactivity and/or Ki-67-LI (Table 3). Using this model, 63 informative cases were classified into low- (n = 37) and high-grade lesions (n = 26). Their recurrence rates were 32% and 58%, and their mortality rates were 3% and 35%, respectively. The results show a significant difference in their recurrence risks and mortality rates. Our data demonstrate that these two groups of lesions behave like low- and high-grade soft tissue sarcomas.

It should be noted that, for reported cases, histological grades were evaluated mainly according to two parameters (the architectural features and cellular atypia) in this study. Mitotic counts were not available for about half of the cases from literatures, and Ki-67-LI was provided in only 21 of the reported cases. For this reason, influence of mitoactivity on clinical outcomes was evaluated separately, with the recurrence rates of the mitotically indolent (< 5/10 HPF) and active lesions (≥ 5/10 HPF) determined to be 27% and 58%, respectively. It is expected that, with the assessment based on all of the six parameters including four major and two adjuvant factors (Table 3), the predicting power of our grading model for tumor recurrence risk would be stronger. This has been reflected in our own cases: the recurrence rates of the low- and high-grade lesions were 25% (1/4) and 100% (5/5), respectively.

Among the three parameters associated with clinical outcomes of extranodal FDC sarcoma, tumor size was shown to be most important. It appears that tumor size is a main factor for predicting its recurrence potential, and histological grade is closely associated with mortality rate mainly in patients with larger tumors (Figures 5 and 6). Considering the independent impact of the size to tumor behaviors and close association between mitoactivity and histological grade, we combined the size and grade fac-

tors, and proposed a model for recurrence risk assessment (Figure 6). By this model, FDC sarcomas were classified into low-, intermediate- and high-risk groups. Recurrence rates in the three groups were 16%, 46% and 73%, and the mortality rates were 0%, 4% and 45%, respectively. This will provide a convenient approach for diagnostic pathologists to predict the clinical outcomes of the tumor more accurately following its surgical resection or even with a biopsy. It should be noted that it is impossible to know exactly whether an FDC sarcoma developed from lymph node or extranodal tissues at advanced stages, as in some of the cases in this study. We believe that this model may also be applied for prognosis assessment of the FDC sarcomas which develop from lymph nodes.

Chan *et al*[3] pointed out that intra-abdominal location was associated with a higher recurrence rate. In this study, the recurrence rates of the intra-abdominal lesions and those from other sites were 49% and 38%, respectively, but their difference did not attain a statistical significance. Interestingly, a lower recurrence rate (25%) and a more favorable clinical course were observed in the hepatic lesions as compared with that of the extrahepatic, abdominal/pelvic tumors (60%). It seems to be true that, for extranodal FDC sarcomas of the conventional type, intra-abdominal lesions recur more frequently than those from other sites. The tumors of the former group were found to be larger at diagnosis (9.5 cm) than the latter lesions (5.3 cm). It is conceivable to ascribe their difference in clinical outcomes to their size difference, considering the fact that abdominal/pelvic FDC sarcomas are frequently concealed and diagnosed later compared with those from other sites including head, neck and some superficial areas.

While its pathogenesis has not been established, several factors were linked to development of FDC sarcoma. Some FDC sarcomas from lymph nodes appear to be associated with hyaline-vascular type Castleman disease[86], and similar changes were also noted in tissues surrounding some rare extranodal tumors[38,87,88]. Several reports have observed FDC proliferation and dysplastic changes in lymphoid tissues with Castleman disease where an FDC sarcoma develops[38,89-91], apparently supporting the hypothesis that FDC sarcoma develops from Castleman disease in a hyperplasia - dysplasia - neoplasia sequence[24]. However, the association was not evident in any lesion of our series.

There have been some data indicative of a link between persistent EBV infection and Castleman disease[92,93]. EBER signals were also demonstrated in some FDC sarcomas, particularly the hepatic and splenic lesions[4-9]. Most of these EBV-positive lesions contain numerous inflammatory cells, including plasma cells and lymphocytes, and dispersed tumor cells, thereby being described as inflammatory pseudotumor-like FDC sarcoma[94]. In this study, EBER was demonstrated in the hepatic inflammatory pseudotumor-like lesion (Case 6), but not in any of the six conventional FDC sarcomas examined. It appears to be true that inflammatory pseudotumor-like FDC sarcomas are related to EBV infection.

Clonal cell composition was described in an extranodal FDC sarcoma[17], but greater efforts are required to elucidate its genetic background and establish its molecular pathogenesis. Nuclear immunoreactivity for p53 protein was noticed fortuitously in a few cases[17,38]. In this series, eight extranodal FDC sarcomas without a background of Castleman disease were tested, and a low-level accumulation of p53 protein was observed in majority of tumor cells. Our data raises a possibility of involvement of the p53-mediated pathway during development or progression of FDC sarcoma. In addition, *P53* exons 5-8, where most of the *P53* gene mutations had been found[19,95], were examined in one of the lesions, but without any mutation demonstrated. Based on these data, we consider that the nuclear immunoreactivity may reflect accumulation of wild-type p53 protein. This phenomenon may be an adaptive reaction responsive to elevated mitotic activity and/or increased stress for cell survival, as observed in other cell types under neoplastic[96] and non-neoplastic conditions[18,97-99].

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COMMENTS

Background

Follicular dendritic cell (FDC) sarcoma is a rare tumor, occurring in lymph nodes and extranodal tissues. It is usually regarded as an indolent tumor with a tendency of local recurrence but a low risk of metastasis. With more cases encountered during pathological practice and reported from literatures, various morphologic and clinical representations were noticed. Because of the rarity of the tumor, assessment of its prognosis remains difficult. Clearly, histological grade of this malignancy remains to be defined and a model is needed to evaluate the recurrence risk of individual tumors within this category.

Research frontiers

While FDC sarcoma is regarded as an indolent tumor, like a low-grade soft tissue sarcoma, a broad spectrum of pathologic phenotypes and more aggressive clinical representations were noticed for this tumor type. Intra-abdominal involvement, elevated mitoactivity, coagulative necrosis and marked cellular atypia were considered potentially helpful predictors of an unfavorable outcome, but a grading model remains to be established. In this study, a total number of 106 extranodal FDC sarcomas, nine new cases and 97 from literatures, were analyzed, histological grades of this malignancy were defined and a model was established for recurrence risk assessment of the tumor.

Innovations and breakthroughs

In this study, data of 106 cases of extranodal FDC sarcoma were extracted and reviewed. Histological criteria for low-grade and high-grade FDC sarcomas were established according to four major parameters including architectural pattern, cellular atypia, mitoactivity and/or Ki-67-labeling index. Using this model, 63 informative cases were divided into low- (n = 37) and high-grade lesions (n = 26). Their recurrence rates were 32% and 58%, and the mortality rates were 3% and 35%, respectively. The results showed a significant difference in their recurrence risks and mortality rates. In addition, nine clinicopathological parameters were analyzed, and large tumor size ($\geqslant$ 5 cm in diameter), high-grade histology and elevated mitotic counts ($\geqslant$ 5/10 HPF) were found to be closely associated with the clinical outcomes. According to the tumor size and histological grade, a model was proposed for recurrence risk assessment. By this model, FDC sarcomas were classified into low-, intermediate- and high-risk groups, and their recurrence rates were 16%, 46% and 73%, and the mortality rates were 0%, 4% and 45%, respectively.

Applications

A grading approach was established for evaluating aggressiveness of extranodal FDC sarcomas, and the lesions were divided into low-grade and high-grade categories. According to the tumor size and histological grade, a model was proposed for recurrence risk assessment. This will provide a convenient procedure for diagnostic pathologists to predict the clinical outcomes of the tumor more accurately following its surgical resection or even with a biopsy. This model may also be applied to the prognosis assessment of the FDC sarcomas which develop from lymph nodes.

Peer review

It is a well written and important paper that likely represents the most thorough examination of a rare sarcoma. The authors are to be congratulated for an important work.

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BRIEF ARTICLE

Rate and yield of repeat upper endoscopy in patients with dyspepsia

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Abstract

AIM: To determine the rate and yield of repeat esophagogastroduodenoscopy (EGD) for dyspepsia in clinical practice, whether second opinions drive its use, and whether it is performed at the expense of colorectal cancer screening.

METHODS: We performed a retrospective cohort study of all patients who underwent repeat EGD for dyspepsia from 1996 to 2006 at the University of California, San Francisco endoscopy service.

RESULTS: Of 24780 EGDs, 5460 (22%) were performed for dyspepsia in 4873 patients. Of these, 451 patients (9.3%) underwent repeat EGD for dyspepsia at a median 1.7 (interquartile range, 0.8-3.1) years after initial EGD. Significant findings possibly related to dyspepsia were more likely at initial (29%) *vs* repeat EGD (18%) [odds ratio (OR), 1.45; 95% confidence interval (CI): 1.20-1.75, $P < 0.0001$], and at repeat EGD if the initial EGD had reported such findings (26%) than if it had not (14%) (OR, 1.32; 95% CI: 1.08-1.62, $P = 0.0015$). The same endoscopist performed the repeat and initial EGD in 77% of cases. Of patients aged 50 years or older, 286/311 (92%) underwent lower endoscopy.

CONCLUSION: Repeat EGD for dyspepsia occurred at a low but substantial rate, with lower yield than initial EGD. Optimizing endoscopy use remains a public health priority.

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Key words: Dyspepsia; Esophagogastroduodenoscopy; Health resources; Diagnostic techniques and procedures; Repeat; Treatment outcome

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INTRODUCTION

Dyspepsia, which can be defined as chronic or recurrent pain or discomfort centered in the upper abdomen, is a highly prevalent condition, affecting approximately 20% of the population in Western countries[1-4]. The major causes of dyspepsia are peptic ulcer disease, gastroesophageal reflux disease, malignancy, and functional dyspepsia[5]. The evaluation and management of dyspepsia constitutes a significant clinical and economic burden[6-8].

Non-invasive strategies, including testing and treatment for *Helicobacter pylori*, are recommended for younger persons without alarm symptoms, while prompt esophagogastroduodenoscopy (EGD) is recommended for older persons and those with alarm symptoms[9-11]. An important aim has been to decrease the use of EGD, which is costly and reveals no abnormalities in the majority of patients with dyspepsia[5]. In randomized, controlled trials in multiple countries, non-invasive strategies for dyspepsia led to EGD within 1 year in 2%-50% of patients[12-17]. In these trials, 5%-25% of patients assigned to prompt EGD underwent repeat EGD within 1 year[13-15,17] reflecting the frequently persistent nature of dyspepsia, the difficulty in managing patients with functional dyspepsia[18,19], and the imperfect reassurance value of a normal endoscopy[20-22].

The rate and yield of repeat EGD for dyspepsia in clinical practice, outside of a controlled trial, is unknown. We performed a retrospective, observational study to determine the rate and yield of repeat EGD for dyspepsia in the endoscopy service of the University of California, San Francisco (UCSF), USA. We examined whether different endoscopists performed the initial and repeat EGDs in order to shed light on whether the performance of repeat EGD could be related to patients seeking a second opinion. Given the benefits of colorectal cancer screening, we determined the rate of lower endoscopy in patients undergoing repeat EGD for dyspepsia in order to explore whether endoscopic resources might have been used more optimally.

MATERIALS AND METHODS

Endoscopic databases and study population

The study was approved by the UCSF Committee on Human Research. The study sites were Moffitt-Long Hospital, a university tertiary care medical center that also serves as a community hospital for many patients, and Mount Zion Hospital and Clinics, an integral part of the University of California, San Francisco system that serves primary, secondary and tertiary care patients. Records were available for all endoscopic reports from February 1996 to June 2006 in an endoscopic database that serves both sites (Pentax EndoPRO, Version 6.5.2, copyright 1998-2005, Pentax Precision Instrument Co., Orangeburg, New York). We have used this database and methods similar to those of this study in previous investigations[23]. The source population for the study consisted of all patients undergoing any EGD at either site during the study period.

Search strategy, data collection, and definitions

The endoscopic database was searched systematically to identify all patients who underwent EGD for the evaluation of dyspepsia. Dyspepsia as an indication was defined as any of the following terms in the database's "Indications" field: "dyspepsia", "abdominal pain", "abdominal pain despite treatment", "abdominal pain suggesting organic disease", and "bloating". All patients who underwent at least 2 EGDs with an indication of dyspepsia during the study period were included. For all studies in which biopsies were performed because of suspected malignancy or Barrett's esophagus, histological diagnoses were confirmed by review of electronic pathology records.

Data were extracted from the endoscopy and pathology reports and entered into matrix format (Excel 2000, Microsoft, Redmond, WA, USA). Demographic data included gender and date of birth. Data characterizing the procedure included procedure date, physician, and procedure sequence for a given patient (first or second EGD, or subsequent EGDs). By definition, all procedures included dyspepsia as an indication. Additional indications were recorded and categorized as abnormal imaging study, anemia or gastrointestinal bleeding, anorexia and/or weight loss, dysphagia, gastroesophageal reflux disease symptoms, nausea and/or vomiting, follow-up of ulcer, follow-up of Barrett's esophagus, cirrhosis-related, diarrhea and miscellaneous. Findings at endoscopy were recorded and categorized as esophagitis, hiatal hernia, esophageal stricture, gastritis, gastric erosions, gastric ulcer, gastric polyps, duodenitis, duodenal erosions, duodenal ulcer, duodenal stricture, biopsy-proven malignancy, a new diagnosis of biopsy-confirmed Barrett's esophagus, and retained food suggesting gastroparesis. A significant finding possibly related to dyspepsia was defined as esophagitis, gastric or duodenal ulcer, or biopsy-proven malignancy. We also performed analyses with a less stringent definition of a significant finding that also included gastric or duodenal erosions.

An additional search was performed to identify which study patients had undergone a sigmoidoscopy or colonoscopy at any point during the study period. The purpose was to determine whether subjects had undergone procedures that could serve as endoscopy-based colorectal cancer screening.

Statistical analysis

Data were imported into SAS v9.1 (SAS Institute, Cary, NC) for analysis. Procedures were subgrouped into those that had dyspepsia as the exclusive indication and those that had dyspepsia and additional indications. Summary descriptive statistics for demographic descriptors, indications, and findings were calculated for the set of first (initial) EGDs, second (repeat) EGDs, and so on. Relatively few subjects underwent 4 or more EGDs.

Analyses specified *a priori* were performed. Endoscopic findings were compared between EGDs that included only dyspepsia as an indication and those that included dyspepsia and additional indications. Procedure dates were compared between initial and repeat EGDs for each subject in order to determine the time elapsed between procedures. Endoscopic findings were compared between initial and repeat EGDs in order to determine whether the rate of significant findings differed between these. Endoscopic findings at repeat EGD were compared between subjects who did and did not have

Table 1 Characteristics of the study population *n* (%)

	Initial EGD (*n* = 451)	Repeat EGD (*n* = 451)
Age (yr, mean ± SD)	53.4 ± 18.8	55.6 ± 19.2
Gender		
Female	285 (63)	285 (63)
Male	166 (37)	166 (37)
Time from first to second EGD [yr, median (interquartile range)]	--	1.7 (0.8-3.1)
Indications		
Dyspepsia exclusively	255 (57)	253 (56)
Dyspepsia and additional indication(s)	196 (43)	198 (44)

EGD: Esophagogastroduodenoscopy.

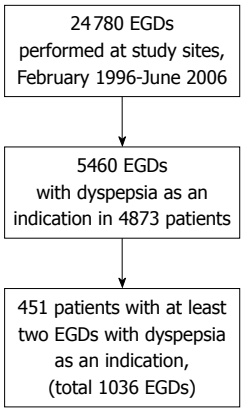


Figure 1 Study population. Starting with all esophagogastroduodenoscopies (EGDs) performed at the study sites during the study period, all EGDs with dyspepsia as an indication were identified, and patients who had undergone at least 2 EGDs with dyspepsia as an indication were selected.

significant findings at initial EGD. Endoscopists performing the initial and repeat EGD were compared to determine whether the same or a different endoscopist performed the procedures.

Continuous variables were compared with the Student's *t*-test, and means with SD are reported. Where appropriate, medians with interquartile ranges are reported. Proportions were compared with the χ^2 test and Mantel-Haenszel odds ratios (OR) with 95% confidence intervals (CI) are reported. Statistical significance was set at P $\alpha \leqslant 0.05$.

RESULTS

Study population

Figure 1 depicts the study population. During the study period, a total of 24780 EGDs were performed at the study sites. Of these, 5460 (22%) included dyspepsia as an indication for the procedure. These 5460 EGDs were performed in 4873 unique patients. A total of 451 of these patients (9.3% of all patients who underwent EGD for dyspepsia) underwent at least 2 EGDs with dyspepsia as an indication, with a total of 1036 EGDs performed with dyspepsia as an indication in this subgroup. The number of patients who underwent 3 or more EGDs with dyspepsia as an indication was 90 having 3 EGDs, 29 having 4 EGDs, 8 having 5 EGDs, 5 having 6 EGDs, 1 having 7 EGDs and 1 having 8 EGDs. Of the 5460 EGDs with dyspepsia as an indication, 585 (10.7%) were second or subsequent EGDs for dyspepsia.

Table 2 Indications for endoscopy *n* (%)

	Initial EGD (*n* = 451)	Repeat EGD (*n* = 451)
Dyspepsia	451 (100)	451 (100)
Dyspepsia	196 (44)	159 (35)
Abdominal pain	168 (37)	199 (44)
Abdominal pain despite treatment	115 (26)	103 (23)
Abdominal pain suggesting organic disease	10 (2)	17 (4)
Bloating	11 (2)	24 (5)
Additional Indications	196 (43)	198 (44)
Abnormal imaging study	1 (0.2)	2 (0.4)
Anemia or gastrointestinal bleeding	27 (6)	15 (3)
Anorexia and/or weight loss	17 (4)	16 (4)
Dysphagia	68 (15)	42 (9)
Gastroesophageal reflux disease symptoms	56 (12)	77 (17)
Nausea and/or vomiting	26 (6)	33 (7)
Follow-up of ulcer	9 (2)	5 (1)
Follow-up of Barrett's esophagus	5 (1)	8 (2)
Cirrhosis-related	1 (0.2)	1 (0.2)
Diarrhea	22 (5)	20 (4)
Miscellaneous[1]	12 (3)	16 (4)

[1]Includes planned dilation, evaluation for graft-versus-host disease, fever, postoperative evaluation, family history of malignancy, and "surveillance".

The demographic and procedure-related characteristics of the study patients are summarized in Table 1. The proportions of EGDs that included dyspepsia as an exclusive indication were nearly identical for the initial and repeat EGDs (56%-57%). Repeat EGD was performed a median of 1.7 (IQR, 0.8-3.1) years after the initial EGD.

Indications for initial and repeat EGD

The indications for the initial and repeat EGD for the patients are shown in Table 2. The distributions of indications classified as "dyspepsia" and those defined as additional indications were each similar for the initial and repeat EGDs.

Findings at initial and repeat EGD

The findings at the initial and repeat EGD for the patients are shown in Table 3. A minority of EGDs showed significant abnormalities. The proportion of EGDs showing a significant finding possibly related to dyspepsia was higher for the initial EGD (133/451, 29%) than for the repeat EGD (79/451, 18%) (OR, 1.45; 95% CI: 1.20-1.75, $P < 0.0001$). There was a higher rate of significant findings possibly related to dyspepsia on the repeat EGD if the initial EGD had reported such findings (35/133, 26%) than if it had not (44/318, 14%) (OR, 1.32; 95% CI: 1.08-1.62, $P = 0.0015$).

The 44 patients who had significant findings only at the repeat EGD had a mean age of 52 (± 23) years at initial EGD, a median time to repeat EGD of 1.8 (IQR, 0.7-4.1) years, and 28/44 (64%) were female. In 27 (61%) of these patients, dyspepsia was the only indication for repeat EGD, while indications in addition to dyspepsia

Table 3 Findings at endoscopy *n* (%)

	Initial EGD (*n* = 451)	Repeat EGD (*n* = 451)
Esophagitis	95 (21)	59 (13)
Hiatal hernia	160 (36)	172 (38)
Esophageal stricture	13 (3)	10 (2)
Gastritis	221 (49)	196 (43)
Gastric erosions	44 (10)	40 (9)
Gastric ulcer	31 (7)	14 (3)
Gastric polyps	21 (5)	21 (5)
Duodenitis	35 (8)	24 (5)
Duodenal erosions	6 (1)	3 (0.7)
Duodenal ulcer	13 (3)	8 (2)
Duodenal stricture	2 (0.5)	3 (0.7)
Retained food suggesting gastroparesis	2 (0.5)	8 (2)
Postoperative changes	11 (2)	18 (4)
Biopsy-proven malignancy	0 (0)	0 (0)
Biopsy-proven Barrett's esophagus (new diagnosis)	3 (0.7)	3 (0.7)
Significant finding possibly related to dyspepsia[1]	133 (29)[2]	79 (18)[2]

[1]Defined as esophagitis, gastric or duodenal ulcer, or biopsy-proven malignancy. The sum of these individual findings in the table is higher than this cumulative value because some patients had more than one individual finding; [2]Odds ratio, 1.45; 95% confidence interval: 1.20-1.75, $P < 0.0001$.

in the other 17 included bleeding, weight loss, nausea and vomiting, diarrhea, dysphagia, and gastroesophageal reflux symptoms. At repeat EGD, the findings were esophagitis in 31 (70%), gastric ulcer in 9 (20%), duodenal ulcer in 3 (7%), and esophagitis and gastric ulcer in 1 (2%). It is not possible to determine whether the findings at repeat EGD were missed at initial EGD.

For the initial EGD, the proportion of tests showing a significant finding possibly related to dyspepsia was higher for procedures with dyspepsia and additional indication(s) than for procedures with dyspepsia as the exclusive indication (36%, or 71/196 *vs* 24%, or 62/255; OR, 1.30; 95% CI: 1.06-1.59, $P = 0.006$). In contrast, for repeat EGD, the proportions of tests showing a significant finding possibly related to dyspepsia did not differ significantly between procedures with dyspepsia and additional indication(s) and procedures with dyspepsia as the exclusive indication (18%, or 36/198 *vs* 17%, or 43/253; OR, 1.04; 95% CI: 0.83-1.29, $P = 0.74$).

With a less stringent definition of a significant finding that also included gastric or duodenal erosions, the proportion of EGDs showing a significant finding possibly related to dyspepsia remained higher for initial EGD (172/451, 38%) than for repeat EGD (111/451, 25%) (OR, 1.40; 95% CI: 1.19-1.65, $P < 0.0001$). With this less stringent definition, there was a trend towards a higher rate of significant findings possibly related to dyspepsia on repeat EGD if initial EGD had reported such findings (51/172, 29%) than if it had not (60/279, 22%) (OR, 1.19; 95% CI: 0.99-1.44, $P = 0.051$).

Physician performing initial and repeat EGD

The repeat EGD was performed by the same physician who performed the initial EGD in 77% (346/451) of the cases.

Sigmoidoscopy or colonoscopy

Of the 451 study patients, 393 (87%) underwent either a sigmoidoscopy or colonoscopy. Among those aged 50 years or older, 286/311 (92%) underwent either a sigmoidoscopy or colonoscopy compared to 107/140 (76%) of those younger than 50 years (OR, 2.09; 95% CI: 1.59-2.75, $P < 0.0001$).

DISCUSSION

In our observational study, over 20% of all EGDs performed included dyspepsia as an indication. Of patients undergoing EGD with dyspepsia as an indication, approximately 9% underwent repeat EGDs for dyspepsia at a median 1.7 years after the initial EGD. This constituted a relatively small but still substantial fraction of endoscopic volume related to dyspepsia. The yield of repeat EGD for dyspepsia was modest, and lower than the yield of initial EGD. For initial EGD, the yield was higher for EGDs performed for dyspepsia and additional indications compared with dyspepsia exclusively. In the vast majority of cases, the repeat EGD was performed by the same endoscopist as the initial EGD, suggesting that the seeking of a second opinion was not a major driver in the rate of repeat EGD. Over 90% of patients aged 50 years or older who underwent repeat EGD for dyspepsia also underwent either sigmoidoscopy or colonoscopy, suggesting that repeat EGD was not undertaken at the expense of tests that can serve for colorectal cancer screening.

Because our study was not prospective and did not capture EGDs performed outside the UCSF endoscopy service, we could not compare our results directly with the rates of repeat EGD for dyspepsia observed in the randomized trials of competing management strategies for uninvestigated dyspepsia. Nonetheless, the fraction of patients undergoing repeat EGD for dyspepsia in our study was within the range of 5%-25% observed in those controlled trials at 1 year[13-15,17], and within the range of 9%-26% observed in follow-up at 6 to 7 years[24,25]. If some patients who underwent an initial EGD for dyspepsia in our study cohort then underwent repeat EGD for dyspepsia at another institution, or if the "initial EGD" for dyspepsia in our study was in reality a repeat EGD, then our results would underestimate the true rate of repeat EGD for dyspepsia. In our study, repeat EGD was performed a median of 1.7 (IQR, 0.8-3.1) years after the initial EGD. Longer-term follow-up of patients in randomized trials has shown that some patients undergo repeat EGD for dyspepsia years later, but most repeat EGDs were within the initial year of the studies[13,24,25].

It is well recognized that most EGDs performed for dyspepsia demonstrate no significant abnormality[5,13-17]. Thus, the relatively low yield in our study was not surprising. The yield at repeat EGD for dyspepsia

was lower than at initial EGD, a finding that might have been expected, but has not been demonstrated in clinical practice previously. Because our study design selected patients with repeat EGD for dyspepsia, the diagnostic yield at initial EGD might actually have been lower than in patients who did not undergo repeat EGD. For instance, no malignancies were diagnosed, but one could postulate that patients diagnosed with malignancy at initial EGD might be less likely to undergo repeat EGD with dyspepsia as an indication.

The management of patients with functional dyspepsia is challenging[18,19]. Patients may seek second opinions in search of underlying disease that may have been missed, or new treatments. We expected to find that a majority of repeat EGDs for dyspepsia had been performed by a different endoscopist than the initial EGDs, but this was not the case. If we failed to capture a significant fraction of all repeat EGDs for dyspepsia in our study patients because they occurred at other institutions, then the proportion of repeat *vs* initial EGDs performed by a different endoscopist would be higher. It is unlikely that the endoscopists in our study played the role of proceduralist instead of consultant, since open-access upper endoscopy is relatively rare in our endoscopy service. One may speculate that repeat EGD in patients with established care with a gastroenterologist could be driven in part by ongoing patient concern[20-22]. We could not address this issue directly in the current study.

With the growing national emphasis on colorectal cancer screening, the capacity and proper utilization of lower endoscopic services have been debated[26-28]. Because we expected that the yield of repeat EGD for dyspepsia would be low, we sought to investigate whether endoscopic services might have been applied better towards colorectal cancer screening in our study patients. We found that a very large majority of our patients also underwent lower endoscopy, at a rate much higher than national uptake rates for colorectal cancer screening[29]. This likely reflected a group of patients who had well established medical care and might in fact utilize more medical resources than the average, as reflected by a high rate of lower endoscopy even in patients younger than 50 years of age. The endoscopic capacity in our study sites is somewhat elastic, so that a repeat EGD for dyspepsia may not directly displace a screening lower endoscopy. We could not determine whether patients outside of our study cohort were denied access to lower endoscopy as a result of the repeat EGDs that were performed. If present at all, this effect would be small. However, this may be a more salient issue in other settings with more constrained resources and different incentive structures for endoscopists.

Our study has limitations. First, because it was a retrospective study without complete coverage of all of our patients' medical contacts, some repeat EGDs at other facilities might have been missed. However, our results accurately reflected the fraction of endoscopic services devoted to initial and repeat EGD for dyspepsia within our study sites. Second, we did not know whether patients whose EGDs were performed for abdominal pain fit the definition of dyspepsia, but we chose to include them because many endoscopists in our unit do no use the term dyspepsia in the indication field, and because we believe that when EGD is performed for abdominal pain it is likely that this pain is in the upper abdomen and can be categorized as dyspepsia. Third, we did not know the management strategies offered to patients, which could have affected the rate of repeat EGD. Fourth, our results may be generalizable to other endoscopy units with similar systems, patients and endoscopists as ours, but not necessarily to those in different practice settings. Fifth, we did not attempt to determine whether lower endoscopies were performed for diagnostic or screening purposes. However, for the purposes of determining resource use in this study, the distinction was not necessary. Finally, it is possible that some patients with abdominal pain and diarrhea had inflammatory bowel disease, and this might have contributed to the use of lower endoscopy, but diarrhea was an additional indication in only 5% of EGDs.

In conclusion, the rate of repeat EGD for dyspepsia in this study was relatively low but still substantial, and it may have underestimated the true rate because of possible repeat EGDs at other facilities. The diagnostic yield of repeat EGD for dyspepsia was modest, and lower than at initial EGD. Our findings do not support the hypothesis that repeat EGD for dyspepsia in our study sites was usually performed for a second opinion by a different endoscopist. Repeat EGD for dyspepsia in our study patients was not performed at the expense of age-appropriate colorectal cancer screening. Optimal allocation of endoscopic resources remains a public health priority.

COMMENTS

Background

Dyspepsia is a highly prevalent condition. Non-invasive strategies to evaluate dyspepsia are recommended for younger persons without alarm symptoms in large part to limit the use of esophagogastroduodenoscopy (EGD). In randomized, controlled trials, 5%-25% of patients assigned to prompt EGD to evaluate dyspepsia underwent repeat EGD within 1 year.

Research frontiers

The rate and yield of repeat EGD for dyspepsia in clinical practice is not known. The optimal use of endoscopic resources is a public healthy priority.

Innovations and breakthroughs

The authors performed a retrospective cohort study of all patients who underwent repeat EGD for dyspepsia from 1996 to 2006 at the University of California, San Francisco endoscopy service. They determined the rate and yield of repeat EGD for dyspepsia, whether second opinions drove its use, and whether endoscopic resource use might have been shifted to colorectal cancer screening.

Applications

Repeat EGD for dyspepsia occurred at a low but substantial rate, and the diagnostic yield was lower than at initial EGD. Because repeat EGD was not usually performed for a second opinion, it is likely that the physician who performed an initial EGD will face the decision as to whether to perform a repeat EGD. In our cohort, repeat EGD was not performed at the expense

of age-appropriate colorectal cancer screening. Nonetheless, optimal use of endoscopic capacity remains a public health priority.

Terminology

Dyspepsia can be defined as chronic or recurrent pain or discomfort centered in the upper.

Peer review

This is an interesting retrospective study to demonstrate the rate and yield of repeat EGD for dyspepsia.

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BRIEF ARTICLE

Efficacy of rituximab in gastric diffuse large B cell lymphoma patients

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Abstract

AIM: To evaluate retrospectively the efficacy of rituximab plus chemotherapy in gastric diffuse large B cell lymphoma (DLBCL).

METHODS: Sixty patients (median age: 58 years) with histologically confirmed gastric DLBCL treated at four Italian institutions between 2000 and 2007, were included in this analysis. Patients were selected by stage (Ⅰ-Ⅳ, Lugano staging system), European Cooperative Oncology Group performance status (0-2) and treatment strategies. Treatment strategies were chemotherapy alone (group A, *n* = 30) [scheduled as cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP) and CHOP-like], and chemotherapy combined with rituximab (group B, *n* = 30). The primary end point of the study was complete response (CR) rate; the secondary end points were disease-free survival (DFS) at 5 years and overall survival (OS).

RESULTS: Median follow-up was 62 mo (range: 31-102 mo). We observed a significant difference between the two groups (A *vs* B) in terms of CR [76.6% (23/30) *vs* 100%, *P* = 0.04) and DFS at 5 years [73.3% (22/30) *vs* 100%, *P* = 0.03). To date, 19 group A (63.3%) patients are alive and 11 have died, while all group B patients are alive. No significant differences in toxicity were observed between the two groups.

CONCLUSION: Rituximab in combination with chemotherapy improves CR rate, DFS and OS. Further prospective trials are needed to confirm our results.

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Key words: Rituximab; Diffuse large B cell lymphoma; Stomach neoplasms; Chemotherapy

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INTRODUCTION

Immunotherapy with rituximab, a chimeric monoclonal antibody against B-cell surface antigen CD20, is widely utilized in non-Hodgkin's lymphoma[1,2]. Rituximab in addition to cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP) chemotherapy represents the gold standard for the treatment of nodal aggressive non-Hodgkin'a lymphoma, since it improves response rate, overall survival (OS) and event-free survival *vs* chemotherapy alone, with no increase in toxicity. These results were obtained in a phase Ⅲ randomized study (GELA) that included a population of 399 previously untreated elderly patients with diffuse large B cell lymphoma (DLBCL)[3].

Primary gastric non-Hodgkin's lymphoma (PGNHL) is the most common extranodal lymphoma, and represents 60%-75% of gastrointestinal lymphoma cases, with an incidence about of 1 per 100 000 in developed countries, which appears to be increasing. PGNHL is more common in men and in individuals aged > 50 years, with a maximum incidence in the seventh decade, but it may also occur in younger patients[4].

Clinical presentation includes a variety of symptoms such as weight loss, anorexia and abdominal pain, whereas gastric bleeding is uncommon. B lymphoma generalized symptoms are less common than in primary nodal lymphoma. All histological lymphoma categories are present, but the main ones are mucosa-associated lymphoid tissue (MALT) lymphoma (low and high grade) and DLBCL[5-7].

The relationship between *Helicobacter pylori* chronic infection and MALT is well known (about 90% of cases), but its role in gastric DLBCL is controversial[4]. Eradication therapy is an effective option for low-grade MALT lymphoma[8].

The CHOP schedule, as a standard treatment for nodal non-Hodgkin's lymphoma, has been utilized in several non-randomized studies and represents an effective option[9,10]. Rituximab and CHOP combination is also commonly utilized in the treatment of gastric DLBCL, but it has only been tested in a few studies[11,12].

Therefore, we carried out a retrospective study to evaluate the efficacy of rituximab in combination with chemotherapy in gastric DLBCL. To date, this study represents the largest analysis.

MATERIALS AND METHODS

In this retrospective study, we analyzed a group of non-gastrectomized patients with gastric DLBCL, who were treated at our four institutions between 2000 and 2007.

Patients

Sixty patients (42 men and 28 women) with a median age of 58 years received only systemic treatment. Performance status according to European Cooperative Oncology Group (ECOG) was 0-2. All patients presented with a histologically confirmed diagnosis of gastric DLBCL. Stages were between I and Ⅳ according to the Lugano staging system (Table 1), with primary localization in the stomach at the antrum (46.6%) and antrum-body (25%). B-symptoms were present in only 14% patients. β2 microglobulin was elevated in 60% of patients and lactate dehydrogenase was elevated in 85.0% (Table 2). The primary end point was the complete response (CR) rate. Secondary end points were disease-free survival (DFS) at 5 years and OS.

Table 1 Lugano staging of GI lymphomas

Stage Ⅰ: tumor confined to GI tract
Stage Ⅱ: tumor extending into abdomen from GI site
Ⅱ 1 = local nodal involvement
Ⅱ 2 = distant nodal involvement
Stage Ⅲ: penetration of serosa to involve adjacent organs or tissue
Stage Ⅳ: disseminated extranodal involvement, or supradiaphragmatic nodal involvement

GI: Gastrointestinal.

Table 2 Clinicopathological characteristics of 60 patients affected by gastric DLBCL

Clinicopathological characteristics	
Age (yr)	
Median	58
Range	19-76
Sex	
Male	42
Female	28
Performance status	
ECOG 0	0 (65%) (39/60)
ECOG 1	1 (28.3%) (17/60)
ECOG 2	2 (3.3%) (17/60)
Primary gastric site	
Body	18.3% (11/60)
Antrum	46.6% (28/60)
Fundus	10% (6/60)
Antrum-body	25% (15/60)
Metastatic sites	
No	23.3% (14/60)
Node	55% (33/60)
Pulmonary	18.3% (11/60)
Bone	0% (0/60)
Liver	1.6% (1/60)
Spleen	0% (0/60)
Pleural effusion	1.6% (1/60)
Bio-humoral parameters	
Lactate dehydrogenase (high level)	85.0% (51/60)
β2 microglobulin (high level)	60% (36/60)
Lugano staging system	
Ⅰ	28.3% (17/60)
Ⅱ	46.6% (28/60)
Ⅲ	16.6% (10/60)
Ⅳ	8.3% (5/60)
B symptoms	15% (9/60)

ECOG: European Cooperative Oncology Group; DLBCL: Diffuse large B cell lymphoma.

Treatment

We selected 60 cases from an archive of patients that had received chemotherapy plus immunotherapy or chemotherapy alone from 2000 to 2007. Thirty of these patients (group A) received only chemotherapy accord-

Table 3 Treatment response

Chemo-immunotherapy treatment	
No. of patients	
CHOP-like	30
CHOP-like and rituximab	30
Second or other lines	7.50%
No. of cycles	
Median	6 (3-24)
mean	8.3 (3-24)
Results	
Median follow-up	77 mo
CHOP-like	CR = 76.6%, DFS at 5 yr = 73.3%, OS = 63.3%
CHOP-like and rituximab	CR = 100%, DFS at 5 yr = 100%, OS = 100%

CHOP: Cyclophosphamide, doxorubicin, vincristine and prednisone; CR: Complete response; DFS: Disease-free survival; OS: Overall survival.

ing to CHOP or CHOP-like (MACOP-B) schedules. The CHOP schedule consisted of cyclophosphamide at 750 mg/m^2 on day 1, doxorubicin 50 mg/m^2 on day 1, vincristine 1.4 mg/m^2 up to a maximal dose of 2 mg on day 1, and prednisone 100 mg/d for 5 d, every 21 d. MACOP-B schedule consisted of methotrexate 100 mg/m^2 on day 1, adriamycin 35 mg/m^2 on day 1, cyclophosphamide 350 mg/m^2 on day 1, vincristine 1.4 mg/m^2 up to a maximal dose of 2 mg on day 1, prednisone 40 mg/m^2 on days 1-5, and bleomycin 10 mg/m^2, every 21 d. A group of 30 patients (group B) received rituximab (375 mg/m^2 administrated on day 1 of each cycle of CHOP or CHOP-like MACOP-B) (Table 3). Rituximab infusion was interrupted in the event of fever, chills, edema, congestion of the head and neck mucosa, hypotension or any other serious adverse event, and it was resumed when the event resolved. If the absolute neutrophil (granulocyte) count was < 1500/μL or the platelet count was 100000/μL, chemotherapy was halted for up to 1 wk, and treatment was stopped. The dose of rituximab was not modified, and rituximab was continued when CHOP was stopped. Treatment was stopped, however, if lymphoma progressed, if the patient declined to continue, or at the discretion of the physicians in cases of concurrent illness or adverse events.

Response to treatment and adverse events

Response to treatment was classified as CR, partial response (PR) or progressive disease (PD) according to the International Workshop criteria[13]. CR was defined as the disappearance of all lesions and radiological or biological abnormalities observed at diagnosis, and the absence of new lesions. PR was defined as regression of all measurable lesions by > 50%, disappearance of non-measurable lesions, and absence of new lesions. PD was defined as the appearance of new lesions, any growth of the initial lesions by > 25%, or growth of any measurable lesion that had regressed during treatment by > 50% from its smallest dimensions. All adverse events reported by the patient or observed by the investigator were collected and were graded according to the NCI toxicity criteria (CTCAE v3.0).

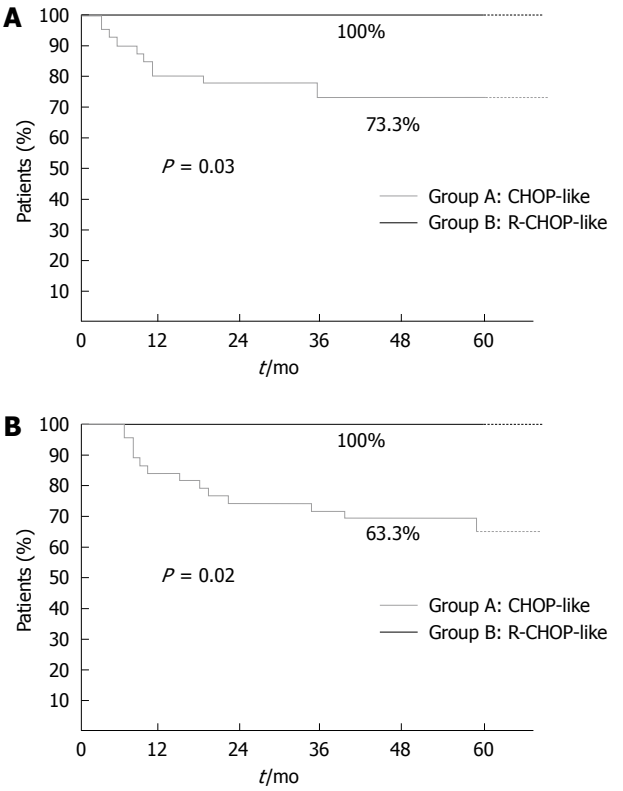


Figure 1 Disease-free survival (DFS) (A) and overall survival (OS) (B) curve. Group A: Cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP); Group B: Rituximab plus chemotherapy.

Statistical analysis

Quantitative data were described by the median (range), and qualitative data were expressed as counts and percentages. Duration of the disease-free interval and OS were estimated according to the Kaplan-Meier method. Fisher's exact test was used to assess the significance of all correlations. Statistical significance was achieved if P was < 0.05. All reported P values were two-sided.

RESULTS

Treatment response

Median follow-up was 62 mo (range: 31-102 mo). No difference between the two groups was observed in term of stage, ECOG performance status and metastatic sites. Rituximab plus chemotherapy *vs* chemotherapy alone yielded a statistically significant advantage in terms of CR (100% *vs* 76.6%, P = 0.004), DFS at 5 years (100% *vs* 73.3%, P = 0.03) (Figure 1A) and OS (100% *vs* 63.3%, P = 0.02) (Figure 1B). We observed in group A, five patients with PR and two with PD. Only 7.5% of patients underwent a second or additional line of therapy (Table 3). To date, 19 group A patients are alive and 11 have died [nine due to lymphoma-related causes, one to brain metastatic melanoma, and one to hepatocarcinoma (hepatitis C virus-related)]. All patients in group B are alive.

Table 4 Toxicity *n* (%)

	CHOP-like			CHOP-like and rituximab		
	G1	G2/3	G4	G1	G2/3	G4
Hematological						
Hemoglobin level	6 (20)	6 (20)	1 (3.3)	7 (23.3)	6 (20)	1 (3.3)
Neutrophil count	8 (26.6)	12 (40)	10 (33.3)	9 (30)	12 (40)	11 (36.6)
Platelet count	4 (13.3)	3 (10)	2 (6.6)	3 (10)	2 (6.6)	1 (3.3)
Gastrointestinal						
Nausea/vomiting	10 (33.3)	4 (13.3)	0 (0)	10 (33.3)	4 (13.3)	1 (3.3)
Diarrhea	3 (10)	2 (6.6)	0 (0)	3 (10)	1 (3.3)	0 (0)
Stomatitis	6 (20)	2 (6.6)	0 (0)	6 (20)	3 (10)	0 (0)
Constipation	3 (10)	1 (3.3)	0 (0)	3 (10)	1 (3.3)	0 (0)
Dysphagia	1 (3.3)	2 (6.6)	0 (0)	1 (3.3)	1 (3.3)	0 (0)
Pyrosis	5 (16.6)	4 (13.3)	0 (0)	6 (20)	3 (10)	0 (0)
Other						
Fatigue	8 (26.6)	6 (20)	1 (3.3)	9 (30)	6 (20)	1 (3.3)
Alopecia	3 (10)	5 (16.6)	14 (46.6)	4 (13.3)	6 (20)	17 (56.6)
Fever	3 (10)	4 (13.3)	1 (3.3)	4 (13.3)	5 (16.6)	1 (3.3)
Allergic reaction	1 (3.3)	1 (3.3)	0 (0)	2 (6.6)	1 (3.3)	0 (0)
Pharyngitis erythema	0 (0)	0 (0)	0 (0)	1 (3.3)	0 (0)	0 (0)
Paresthesia	9 (30)	2 (6.6)	0 (0)	7 (23.3)	3 (10)	1 (3.3)
Arthromyalgia	1 (3.3)	1 (3.3)	0 (0)	1 (3.3)	1 (3.3)	0 (0)
Recurrent infections	6 (20)	4 (13.3)	1 (3.3)	7 (23.3)	5 (16.6)	1 (3.3)
Thrombosis	2 (6.6)	1 (3.3)	0 (0)	2 (6.6)	1 (3.3)	0 (0)
Cough	2 (6.6)	2 (6.6)	1 (3.3)	3 (10)	2 (6.6)	0 (0)
Pain	1 (3.3)	1 (3.3)	0 (0)	2 (6.6)	1 (3.3)	0 (0)

Toxicity

The most common toxicities were grade 2/3 and 4 granulocytopenia (76.6% in group B and 73.3% in group A, $P > 0.05$) and recurrent infection (all grades) (43.2% in group B and 36.6% in group A, $P > 0.05$) (Table 4).

DISCUSSION

Our analysis is believed to be the first study to compare directly chemo-immunotherapy and chemotherapy alone in gastric non-Hodgkin's lymphoma. We demonstrated a statistically significant improvement with chemo-immunotherapy in terms of CR, DFS at 5 years and OS, without a significantly greater toxicity.

Rituximab was introduced as a treatment for prior nodal lymphoma at the end of the 1990s. A randomized phase Ⅲ trial (GELA group) has underlined the effectiveness of rituximab in prior nodal B cell lymphoma. Three hundred and ninety-nine previously untreated elderly patients (≥ 60 years) with stage Ⅱ-Ⅳ DLBCL were randomized to receive either standard CHOP or rituximab plus CHOP for eight cycles. CR rate was significantly higher in the group that received rituximab plus CHOP than in the group that received CHOP alone (76% *vs* 63%, $P = 0.005$), with a median follow-up of 2 years, DFS and OS times were significantly longer in the rituximab plus CHOP group ($P < 0.001$ and $P = 0.007$, respectively). Clinically relevant toxicity was not significantly greater with rituximab plus CHOP[3].

However, as far as we are aware, no previous studies have compared immunotherapy-chemotherapy with chemotherapy alone in gastric DLBCL.

A few studies have evaluated immuno-chemotherapy in gastric DLBCL. In one single-arm study, 15 patients were treated with rituximab plus chemotherapy. CR rate was 87% and PR rate was 13%. The CR rate was high in this study, as in our analysis, but there was no direct comparison with chemotherapy alone[11]. A phase Ⅱ trial of 42 patients with early-stage disease did not show any advantage of rituximab plus CHOP chemotherapy. A CR was obtained in 95% of cases, and relapses were observed in two[12]. The treatment did not achieve a statistically significant effect in terms of DFS at 5 years and OS at 5 years in comparison with the historical control[14].

Our results are in agreement with this previous study in term of CR, and confirmed that the chemotherapy-immunotherapy was more active than chemotherapy alone.

Our study had several limitations. First, it was a retrospective study with potential bias in the patients and methods. Second, chemotherapy was not the same in each group (the first patients were treated mainly with the MACOP-B schedule, while the more recent patients were treated mainly with the CHOP schedule).

In conclusion, our results are promising in terms of CR, DFS and safety. However, prospective randomized trials are needed to confirm these preliminary results.

COMMENTS

Background

Rituximab in addition to cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP) chemotherapy represents the gold standard for the treatment of nodal aggressive non-Hodgkin's lymphoma.

Research frontiers

Rituximab and CHOP combination is also commonly utilized in the treatment of gastric diffuse large B cell lymphoma (DLBCL), but it has only been tested in a few studies. Therefore, the authors carried out a retrospective study to evaluate the efficacy of rituximab in combination with chemotherapy in gastric DLBCL.

Applications

To date, this study represents the largest analysis. The analysis represents the first study to compare directly chemo-immunotherapy and chemotherapy alone in gastric non-Hodgkin's lymphoma. The results are promising in terms of CR, DFS and safety. However, prospective randomized trials are needed to confirm these preliminary results.

Terminology

Rituximab is a chimeric monoclonal antibody against B-cell surface antigen CD20 and it is widely utilized in non-Hodgkin's lymphoma therapy. Primary gastric non-Hodgkin's lymphoma is the most common extranodal lymphoma, and represents 60%-75% of gastrointestinal lymphoma cases. The main histological types are mucosa-associated lymphoid tissue lymphoma (low and high grade) and DLBCL. The CHOP schedule consists of cyclophosphamide, doxorubicin, vincristine and prednisone.

Peer review

The study is interesting as it is the first to compare the two types of therapy in these gastric tumors.

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BRIEF ARTICLE

Nonalcoholic fatty liver disease and *HFE* gene mutations: A Polish study

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Abstract

AIM: To describe a Polish population with nonalcoholic fatty liver disease (NAFLD) with regard to *HFE* gene mutations, as well as analyzing demographic and clinical data.

METHODS: Sixty-two consecutive patients with biopsy-proven NAFLD were included in the study. Demographic, clinical, and laboratory data were summarized in a database. C282Y and H63D mutations of the *HFE* gene were analyzed using polymerase chain reaction-restriction fragment lenght polymorphism.

RESULTS: The analyzed cohort consisted of 62 homogeneic Caucasian participants, 66.1% men and 33.9% women, with a median age of 48 years. The median body mass index was 29.05 kg/m^2. Hypercholesterolemia was observed in 74.2% of patients and hypertriglyceridemia in 32.2%; 16.1% had type 2 diabetes mellitus (DMt2). On liver biopsy, 22.6% of NAFLD patients were found to have severe fibrosis. There were no differences between frequencies of *HFE* gene mutations in subgroups of NAFLD patients with less and more severe liver fibrosis. Obesity, older age, female gender and DMt2 were associated with more advanced fibrosis in this Polish cohort, as well as higher glucose level, serum iron and transaminase aspartate aminotransferase/alanine aminotransferase ratio.

CONCLUSION: *HFE* mutations conferred no additional hepatic fibrosis risk in NAFLD, but higher serum iron was a risk factor for severe liver damage in NAFLD, regardless of *HFE* mutations.

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Key words: Nonalcoholic fatty liver disease; *HFE* gene mutations; Liver fibrosis

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is now the most common liver disease in Western countries, affecting up to a third of individuals. It covers a spectrum of liver problems including benign simple steatosis (fatty liver) and steatohepatitis [nonalcoholic steatohepatitis (NASH)] with hepatic injury, inflammation, and fibrosis, which can lead to cirrhosis, liver failure, and hepatocellular carcinoma[1]. NAFLD is strongly associated with metabolic syndrome and is now regarded as its liver manifestation. Hyperinsulinemia is a risk factor for the development of NASH. A moderate increase of iron stores is observed in NASH, and its presence may contribute to the development of insulin resistance (IR)[2,3].

Some studies have identified high ferritin level and/or increased prevalence of hyperferritinemia in patients with metabolic syndrome[4] or its single components[5]. Serum ferritin could be a marker of IR[6]. Increased ferritin levels, together with high transferrin saturation, are typical features of *HFE* hereditary hemochromatosis (HHC). However, hyperferritinemia with normal or slightly increased transferrin saturation has also been described in non-C282Y homozygotes with NASH and has been designated "IR hepatic iron overload syndrome" because of a frequent association with hepatic steatosis and metabolic abnormalities[7].

The mechanism determining the progression from fatty liver to steatohepatitis is still unclear, and there is a conflict of evidence regarding inheritance of the *HFE* gene mutation and the influence of hepatic iron deposition as cofactors for developing fibrosis in NAFLD. Family studies and inter-ethnic variations in susceptibility suggest that genetic factors may be important in determining disease risk or the clinical course of the disease. Thus, the aim of this study was to evaluate the frequency of the *HFE* gene polymorphisms C282Y and H63D in Polish patients with NAFLD with respect to demographic, clinical and laboratory data.

MATERIALS AND METHODS

Sixty-two consecutive patients with biopsy-proven NAFLD were included in the study between 2007 and 2009. Patients were recruited consecutively by means of liver biopsy. We analyzed only the patients with a negative history of alcohol intake; those consuming more than 20 g/d were excluded from the study. All patients tested negative for HBV antigen and anti-HCV antibodies. Those aged 40 years or less had their ceruloplasmin checked and confirmed to be normal. γ-globulins, immunoglobulins, and auto-antibodies were tested and imaging diagnostics were performed to exclude autoimmune hepatitis as well as primary biliary cirrhosis and primary sclerosing cholangitis. Advanced fibrosis in liver biopsy was defined as bridging fibrosis F3 and cirrhosis F4. Demographic and clinical data were summarized in a database.

HFE gene analysis was performed by polymerase chain reaction (PCR) amplification of total genomic DNA of two regions of the gene carrying the mutations C282Y and H63D. The PCR primers for amplifications of the C282Y locus were 5'-TCCGTCTTAGCTGAGTGGAACTACTACCCCCAGAACATCACC-3' and 5'-AGGCAGAATCGACTCACCTGGCTCTCATCAGTCACATACCC-3'. For detection of H63D, the sense primer sequence was 5'-ATGGTTAAGGCCTGTTGCTCTGTC-3' and the antisense primer sequence was 5'-CCCTTGCTGTGGTTGTGATTTTC-3'. PCR amplification was performed in a total volume of 12.5 μL containing 10 to 20 ng genomic DNA, 2.5 nmol of each deoxynucleotide triphosphate, 4 pmol of each primer, 1.5 mmol/L magnesium chloride, 1 × PCR buffer solution, and 0.3 U *Taq* polymerase (MBI Fermentas, Vilnius, Lithuania). PCR amplification consisted of initial denaturation for 3 min at 94℃, followed by 35 cycles of denaturation at 94℃ for 30 s, annealing at 56℃ for 30 s, and extension at 72℃ for 30 s. The terminal elongation was performed at 72℃ for 7 min.

PCR products were digested with *Rsa* I (MBI Fermentas, Vilnius, Lithuania) for detection of C284Y and with *Mbo* I (MBI Fermentas, Vilnius, Lithuania) for H63D. The G-to-A transition (aa282) creates a new *Rsa* I site, and a second *Rsa* I site in this fragment acts as internal control within the restriction fragment length polymorphism. The C-to-G transversion (aa63) results in loss of one of two *Mbo* I sites in the amplified product. DNA fragments generated after digestion were separated on 3% agarose gels.

Statistical analysis

The statistical analysis was performed using SPSS v 15.0. The independent-samples *t* test, preceded by Levene test for equality of variances test, was used to compare means in continuous data. Pearson χ^2 test was used to check the association with advanced fibrosis. The Pearson correlation coefficient and Spearman rho coefficient of correlation were also calculated. Multivariate logistic regression was used to determine the set of data independently correlated with advanced fibrosis.

RESULTS

The analyzed cohort consisted of 62 racially homogeneic Caucasian patients. There was a predominance of males over females (66.1% *vs* 33.9%). The median age of patients was 48 years (ranges, women: 30-75 years; men: 25-64 years). Body mass index (BMI) in women ranged from 21.32 to 39.3 kg/m^2 and in men from 21.9 to 37.7 kg/m^2. More than half of the analyzed group was obese (BMI > 30 kg/m^2 in 50.4%), and 38.1% of patients were overweight (25 kg/m^2 < BMI < 30 kg/m^2).

Table 1 Selected characteristics of patients with NAFLD

	Minimum	Median	Maximum	mean ± SD
Age (yr)	25	48	75	46.56 ± 11.91
Fe (10.56-228.3 μmol/L)	4.12	20.9	68.4	21.4 ± 9.79
Transferrin saturation (< 45%)	7.6	36	206	40.59 ± 30.46
Ferritin (629.2-7999.3 pmol/L)	629.2	5336.6	129584.4	9714.9 ± 18409.2
AST (< 38 IU/L)	16	45.5	275	57.44 ± 43.91
ALT (< 41 IU/L)	15	73	281	86.37 ± 57.35
AST/ALT ratio	0.36	0.62	2.43	0.79 ± 0.47
PLT (150-400 × 10^9/L)	93	218	376	224.98 ± 67.47
Albumin (34-48 g/L)	36.8	46.2	54.8	45.8 ± 4.0
Glycemia (3.89-5.83 mmol/L)	3.44	5.6	11.8	581.7 ± 1.27
BMI (19-25 kg/m^2)	21.2	29.05	39.3	29.10 ± 4.17
HOMA (> 1.8)	0.44	4	35.63	7.44 ± 9.25
HBA_{1c} (4.8%-5.9%)	4.99	5.76	9.76	6.20 ± 1.26

NAFLD: Nonalcoholic fatty liver disease; Fe: Iron; AST: Aspartate transaminase; ALT: Alanine transaminase; PLT: Platelet count; BMI: Body mass index; HOMA: Homeostatic model assessment; HBA_{1c}: Glycated hemoglobin.

Among co-morbidities, hypercholesterolemia (total cholesterol > 5.18 mmol/L) was present in 74.2% of patients; hypertriglyceridemia (> 2.03 mmol/L) in 32.2%; type 2 diabetes mellitus (DMt2) in 16.1%; and arterial hypertension in 21.0%. Selected demographic and clinical data are presented in Table 1.

Among the 62 participants, 14 patients (22.6%) had severe (F3-F4) fibrosis and 48 (77.4%) had no or mild fibrosis. Table 2 summarizes the clinical data related to NASH with no/mild (F0-F2) and advanced (F3-F4) fibrosis.

High serum aspartate aminotransferase/alanine aminotransferase ratio, and higher serum iron were strongly associated with advanced fibrosis (F3-F4). Lower levels of albumin were also indicators of more severe liver disease. There were no statistically significant interactions between gender and common co-morbidities and NAFLD, although a trend was observed in a subgroup of patients with F3-F4 fibrosis and female gender as well as DMt2. The Spearman's correlation coefficients showed higher age, higher BMI and presence of DMt2 as being associated with more advanced fibrosis. Multivariate logistic regression was provided and evidenced DMt2, higher age and female gender as good predictors of fibrosis F3-F4.

Table 3 shows the frequencies of the *HFE* gene mutations C282Y and H63D. There were no statistically significant differences between frequencies of mutated alleles in the subgroup of patients with NAFLD. The number of mutated alleles was too low to identify a Pearson correlation between lipids, iron, ferritin, and transferrin saturation or clinical course (i.e. severe fibrosis) and *HFE* gene mutations. However, a trend towards statistically significant correlations between at least one mutated allele of the H63D *HFE* gene and total cholesterol ($P = 0.034$) as well as LDL-cholesterol ($P = 0.020$) was observed in the group of NASH patients with F3-F4 fibrosis with the Pearson correlation coefficient.

Table 2 Comparison of selected clinical data between patients with fatty liver and no/mild and advanced fibrosis

	Fibrosis 0-2 (n = 48)	Fibrosis 3-4 (n = 14)	P value
Age (yr)	45 ± 12	51 ± 11	0.096
BMI (19-25 kg/m^2)	28.68 ± 3.85	30.64 ± 5.05	0.135
AST (< 38 IU/L)	51.27 ± 38.75	80.69 ± 55.27	0.091
ALT (< 41 IU/L)	89.10 ± 53.64	75.85 ± 71.39	0.462
AST/ALT ratio	0.65 ± 0.31	1.33 ± 0.55	0.001
PLT (150-400 × 10^9/L)	231.08 ± 66.51	201.54 ± 68.57	0.161
Glucose (3.89-5.83 mmol/L)	5.36 ± 0.74	7.08 ± 20.5	0.020
HOMA (> 1.8)	6.0 ± 8.4	13.1 ± 10.7	0.144
Fe (10.56-228.28 μmol/L)	19.9 ± 7.32	26.9 ± 15.0	0.028
Ferritin (629.2-7999.3 pmol/L)	742.2 ± 753.9	1934.4 ± 3914.3	0.362
Albumin (34-48 g/L)	4.64 ± 0.38	4.31 ± 0.37	0.017
HBA_{1c} (4.8%-5.9%)	5.92 ± 0.96	7.48 ± 1.78	0.121
Transferrin saturation (< 45%)	38.68 ± 30.88	49.06 ± 28.64	0.361

Table 3 Frequencies of C282Y and H63D *HFE* gene mutations in patients with NAFLD

HFE gene mutation	Fibrosis 0-2 (n = 48)	Fibrosis 3-4 (n = 14)
C282Y/C282Y	1	0
C282Y/W	1	0
H63D/H63D	3	0
H63D/W	11	3

DISCUSSION

NAFLD has become the most common liver disorder of our times. In about 15% of all NAFLD cases, steatosis may evolve into steatohepatitis, a mix of inflammation, hepatocellular injury and fibrosis, often resulting in cirrhosis and even hepatocellular carcinoma, but this process is not well understood[8]. The pathogenesis of NAFLD is often posited as the "double-hit" hypothesis, in which hepatocellular lipid accumulation is the first hit, followed by a second hit in which pro-inflammatory mediators and reactive oxygen species induce inflammation, hepatocellular injury, and fibrosis. Altered abundance and composition of liver tissue lipids may impair sinusoidal perfusion and modulate the microvascular inflammatory response[8]. On the other hand, in chronic liver disease, iron deposits are found in hepatocytes and in Kupffer/sinusoidal cells, leading to oxidative stress, cell toxicity, and genotoxicity[2]. Despite a number of reports supporting a hypothesis of an association among ferritin, iron overload, IR, and NAFLD, the mechanisms underlying this intricate and intriguing relationship remain unclear, but a likelihood is that IR, fatty acid accumula-

tion, and oxidation are the major determinants of serum ferritin levels[9].

The goal of this study was to describe a racially homogeneic cohort of Polish patients with NAFLD with respect to *HFE* gene mutations as well as their demographic, clinical, and laboratory data. The results of our study showed similar clinical and laboratory markers to those previously described as being associated with advanced fibrosis[1]. The Polish NAFLD population differs with regard to two points: our patients are less obese than the patients participating in Harrison *et al*[10] and Angulo *et al*[11] and only 16.1% of Polish patients in the current work had diabetes mellitus. In these two US studies - the largest cohort studies in NAFLD - the participants had higher BMI and DMt2 affected 35%[10] and 30%[11] of individuals, respectively.

The frequency of *HFE* gene mutations in the Polish NAFLD population is similar to the frequencies in a healthy Polish cohort[12] and other Central European countries[12] as well as in 119 Polish patients with alcoholic liver disease[13]. The first report about the connection between *HFE* gene mutations and NAFLD showed a positive correlation between levels of serum ferritin, iron, and transferrin saturation and the presence of the mutated allele, as well as between C282Y mutation and more severe fibrosis in a North American population[14]. The results of George *et al*[15] supported these findings, but subsequent larger series have not confirmed such an association[3,16-18]. The results of Chitturi *et al*[18] even brought into question the link between hepatic iron and liver fibrogenesis, and both Adams *et al*[16] and Bugianesi *et al*[3] in their series reported a lack of an association between hepatic steatosis and hepatic fibrosis in patients with HHC and iron overload. On the other hand, among patients homozygous for the C282Y *HFE* gene mutation, the degree of hepatic iron loading remains the dominant factor associated with liver fibrosis[16]. Serum ferritin is often raised in NAFLD patients[3,9] and has been associated with advanced fibrosis[18]. In our Polish cohort we also found the highest serum ferritin levels in the subgroup of NASH patients with F3-F4 fibrosis, although without statistical significance.

In the current study, higher levels of serum iron, as well as DMt2, were associated with severe liver fibrosis. There is a growing body of work showing that iron and glucose metabolism are interdependent and that an increased iron store may contribute to IR. Serum iron may contribute to IR *via* increased adipocyte lipolysis and impairment of glucose transport[19]. There is evidence from *in vitro* studies that iron can reduce binding of insulin to its receptor and reduce insulin receptor gene expression[20]. Valenti *et al*[21] showed that higher iron stores were associated with more advanced liver fibrosis.

The conflicting evidence regarding *HFE* gene mutation and NAFLD could be the result of the ethnic structure of a cohort study. The results from our study with a racially homogeneic Caucasian population showed no increased frequency of mutated alleles of the *HFE* gene in Polish NAFLD patients as well as no association between mutations and iron, ferritin, and transferrin saturation. In addition, no connection was found between *HFE* gene mutation and the clinical course of NAFLD, supporting the results of Bugianesi *et al*[3] and Yoneda *et al*[22]. An increased ferritin level is a marker of severe histological liver damage in some studies[3,9] and also in ours such a trend was observed. Together with the lack of an increased transferrin saturation and increased serum iron independently of *HFE* gene mutations, the results point strongly towards IR hepatic iron overload as a cause of progression in NAFLD. Data obtained in studies of NAFLD provide the possible mechanism of iron interfering with lipid metabolism[23] and thereby promoting hyperglycemia. There is a suggestion that the effect of iron on steatosis development may extend to other liver diseases[24].

Our study provides novel findings regarding *HFE* gene mutations and cholesterol levels. We found that in a subgroup of NASH patients with severe fibrosis, there were statistically significant correlations between the presence of at least one mutated allele of H63D and total cholesterol as well as LDL-cholesterol levels. Although H63D *HFE* gene mutations are quite common in the general population from the northern part of Europe, these results contrast with those of Pankow *et al*'s[25] US population-based study, but support our findings from a previous alcoholic liver disease study[13]. These novel results may be of value in explaining progressive liver fibrosis in at least some proportion of patients with NAFLD. Presence of *HFE* H63D mutation with elevated total and LDL cholesterol, together with increased iron levels, may lead to excessive free fatty acid oxidation with free oxygen radical formation, increased inflammation, and fibrogenesis. However, this novel aspect of NAFLD pathogenesis should be evaluated in a larger cohort of patients, because a previous study with Caucasians showed higher prevalence of the C282Y mutation in NAFLD[26]. In other studies, the frequencies of C282Y *HFE* gene mutation were similar in NAFLD patients and in the general population[27-29] and none of them found increased prevalence of H63D mutation in NAFLD.

In conclusion, this Polish population of patients with NAFLD was similar to populations described in the largest cohort studies from the United States, according to demographic and clinical data, although our patients were less obese and with no incidence of morbid obesity among them. The risk factors for advanced fibrosis in this Polish NAFLD cohort are also approximately similar to those previously reported. In addition, the frequency of *HFE* gene mutations was similar to that described for populations in Central-Eastern Europe, and neither the C282Y nor the H63D *HFE* gene mutations were associated with NAFLD pathogenesis in this cohort. Our study results also suggest a novel possible pathogenesis in some populations of NAFLD patients; however, these results should be validated in larger and racially heterogeneic populations of NAFLD patients.

COMMENTS

Background

Nonalcoholic fatty liver disease (NAFLD) is now the commonest liver disease, affecting up to a third of individuals. It includes a wide spectrum of liver diseases, ranging from pure steatosis to nonalcoholic steatohepatitis (NASH), and can eventually lead to liver cirrhosis, liver failure and hepatocelullar carcinoma. Identifying the biochemical markers and gene mutations leading to more severe liver damage in patients with NAFLD is crucial for evaluating their prognosis.

Research frontiers

There is conflicting evidence regarding inheritance of *HFE* gene mutations [hereditary hemochromatosis (HHC)] as a cofactor for development of more severe fibrosis in NAFLD patients. Although serum ferritin is an independent predictor of hepatic fibrosis, the mechanisms underlying the intricate and intriguing relationship with NAFLD are still unclear. The authors analyzed different biochemical and clinical parameters of sixty two patients with biopsy-proven NAFLD. *HFE* gene analysis was performed by polymerase chain reaction amplification of total genomic DNA of two regions of the *HFE* gene carrying the mutations C282Y and H63D.

Innovations and breakthroughs

Higher serum iron level is a predictor of more advanced fibrosis, in addition to the factors of obesity, older age, female gender and type 2 diabetes mellitus. This research provides novel findings regarding *HFE* gene mutations and cholesterol level. It was found that in a subgroup of NASH patients with severe fibrosis there were correlations between at least one mutated allele of H63D and total cholesterol as well as LDL-cholesterol levels.

Applications

These findings may be of value in the explanation of progressive liver fibrosis in at least some proportion of patients with NAFLD. Higher total and LDL cholesterol levels, together with increased iron levels, may lead to excessive free fatty acid oxidation with free oxygen radical formation, increased inflammation and fibrogenesis. However, this novel aspect of NAFLD pathogenesis should be evaluated in larger cohorts of patients.

Terminology

HFE gene mutation/HHC: An autosomal recessive genetic iron storage disorder, that usually results from defects in this gene. The mutation or polymorphism most commonly associated with hemochromatosis is C282Y. About 1/200 of people of Northern European origin have two copies of this variant; males are particularly at high risk of developing hemochromatosis. Organs commonly affected by hemochromatosis are the liver, heart and endocrine glands.

Peer review

The authors described a Polish population with NAFLD and tried to analyze the correlation of different biochemical and clinical parameters with the disease. This can be considered a very well-designed study.

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BRIEF ARTICLE

Anti-inflammatory efficiency of levobupivacaine in an experimental colitis model

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Abstract

AIM: To investigate the efficiency of levobupivacaine in treating experimentally induced colitis in rats.

METHODS: Colitis was induced by trinitrobenzene sulfonic acid and ethanol in 30 rats under general anesthesia, and 10 rats were used as a sham group. Subsequent to induction of colitis, rats were divided into three groups; budesonide group received 0.1 mg/kg budesonide, levobupivacaine group received 10 mg/kg levobupivacaine and saline group received 1 mL saline solution *via* rectal route for 7 d. In the sham group, only routine rectal catheterization was performed without use of any material. At the end of 7 d, laparotomy and total colectomy were performed for histopathological examination in all rats and blood samples were drawn for measurement of tumor necrosis factor (TNF)-α and interleukin (IL)-6 following cardiac puncture. Macroscopic and microscopic evaluations of the specimens were performed by a pathologist blinded to group assignment of the rats.

RESULTS: Weight loss (P = 0.016) and macroscopic examination scores (P = 0.001) were significantly higher in saline group than others. Histopathological scoring was comparable between all colitis groups (P = 0.350). There was no significant difference in TNF-α levels and IL-6 levels (P = 0.150).

CONCLUSION: The significant improvement in macroscopic scores suggests that levobupivacaine may have topical anti-inflammatory effects in an experimental colitis model; however, this finding was not supported by microscopic findings.

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Key words: Trinitrobenzene sulfonic acid; Colitis; Levobupivacaine; Budesonide

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INTRODUCTION

Inflammatory bowel disease (IBD), usually referring to

ulcerative colitis or Crohn's disease, has an unknown etiology[1]; however, some theories have been proposed. One of the key factors is the excessive immune response of the host[2], which has led to broad use of corticosteroids[3]. Potentially serious side effects of corticosteroids are the most important limiting factor[4]. The autonomic imbalance between sympathetic and parasympathetic nerves is anticipated as another etiological factor[5]. Adrenergic preponderance had been found to be associated with increased epithelial turnover and vasoconstriction resulting in mucosal injury[6]. The beneficial effects of nicotine on IBD support this theory[7].

Local anesthetics, particularly lidocaine, have inhibitory effects on hyperactive autonomic nerves and on host immune response[8-10]. When all these effects are put together, it is not surprising that local lidocaine was effective in the treatment of experimentally produced IBD[11]. Ropivacaine, another local anesthetic with longer acting time than lidocaine had a more pronounced protective effect on mucosal damage when it was topically applied to colonic mucosa in comparison to 5-amino salicylic acid (5-ASA)[12,13]. In this study, we investigated the potential of levobupivacaine, which is a novel, long lasting local anesthetic with less systemic side effects[14], in the treatment of experimentally induced IBD.

MATERIALS AND METHODS

All procedures in this study were approved by local animal ethics committee. The histopathological examinations and biochemical studies were performed, respectively in research laboratories of pathology and biochemistry at Uludag University School of Medicine.

Forty male Sprague-Dawley rats weighing 270 to 390 g were used in this study. The animals were kept in a restricted access room with controlled temperature and light cycle. All rats were housed in individual standard cages. *Ad libitum* standard pellet food and water were maintained for all animals to provide minimum stress.

Induction of colitis

At the day of induction all rats were anesthetized with intramuscular ketamine (8 mg/kg, Ketalar®, Pfizer Inc.) and xylazine (90 mg/kg, Ronpum®, Bayer AG). A 6F feeding tube was inserted rectally until the tip was 8 cm proximal to the anus. The mixture of 0.6 mL of trinitrobenzene sulfonic acid (TNBS) (5% w/v, 40 mg, Sigma Chemical Co., St. Louis, USA) and 0.25 mL of 50% ethanol was instilled into the lumen *via* a feeding tube. Finally, 0.5 mL of air was given to ensure that the whole mixture was instilled into the lumen of the colon.

There were four groups consisting of 10 rats for each: (1) Sham group: no colitis was induced; only rectal insertion of feeding tube was performed once a day from day 1 to day 7; (2) Budesonide group: rats were treated with daily rectal single dose of budesonide (0.1 mg/kg, Entocort® enema, AstraZeneca) *via* feeding tube for 7 d following the induction of colitis; (3) Levobupivacaine group: rats were treated with daily rectal single dose of levobupivacaine (10 mg/kg, Chirocaine®, Abbott) *via* feeding tube for 7 d following the induction of colitis; and (4) Saline group: rats were treated with daily rectal single dose of saline (1 mL, 0.9% NaCl) *via* feeding tube for 7 d following the induction of colitis.

All rectal tube applications were performed under anesthesia.

All rats were weighed daily during the study period. At the end of 7 d, laparotomy and total colectomy after inspection for adhesions were performed for all rats under general anesthesia. Finally, all rats were sacrificed by intra-cardiac puncture to get blood samples for interleukin (IL)-6 and tumor necrosis factor (TNF)-α analysis.

When tissue samples were obtained, macroscopic

Table 1 Macroscopic scoring of mucosal damage in colitis[15]

Macroscopic damage	Score
Ulceration and inflammation	
None	0
Local hyperemia, no ulcer	1
One site of ulcer not accompanied by congestion or thickening of the intestinal wall	2
One site of ulcer accompanied by inflammation	3
⩾ 2 sites of ulcer accompanied by inflammation	4
1 cm > inflammed segment + ulcer site ⩾ 2	5
1 cm < inflammed segment + ulcer site ⩾ 2	6
2 cm ⩽ inflammed segment (the score increases 1 with each 1 cm enlargement of the inflammed segment)	7-10
Adhesions	
None	0
Mild (easy to seperate colon from other tissues)	1
Severe	2
Diarrhea	
None	0
Present	1

Table 2 Microscopic scoring of colitis[15]

Histological lesion	Score
Ulcer	
None	0
Ulcer < 3 mm	1
Ulcer > 3 mm	2
Inflammation	
None	0
Mild	1
Severe	2
Granuloma	
None	0
Present	1
Depth of the disease	
None	0
Submucosal layer	1
Muscular layer	2
Serosal layer	3
Fibrosis	
None	0
Mild	1
Severe	2

Table 3 Comparison of study groups

	Sham	Budesonide	Levobupivacaine	Saline	*P*
Weight loss	10 (0-20)	10 (0-25)	10 (0-20)	0 (0-10)	0.016
Macroscopic damage score	0 (0-1)	1(0-4)	2 (0-5)	4.5 (2-9)	0.001
Microscopic damage score	0 (0-0.66)	1.33 (0-3.33)	0 (0-5.66)	1.66 (0-4.33)	0.014
IL-6	0 (0-38.59)	37.6 (20.3-85.4)	32.3 (0-43.8)	31.9 (7.7-144.8)	0.004
TNF-α	0.1 (0-3.7)	0.5 (0.2-45.7)	0.2 (0.1-312)	0.5 (0.2-255.9)	0.152

TNF: Tumor necrosis factor; IL: Interleukin.

damage was scored on a scale of 0 to 13 modified from a description by Wang *et al*[15] (Table 1) by the same pathologist who was blinded to the group assignment of the rats. Later, the tissue samples were fixed with 10% formaldehyde solution. Tissue samples of 5 mm in length were taken from proximal, middle and distal segments of total colectomy specimens. All samples were embedded in paraffin wax and sections were taken and stained with hematoxylin-eosin (HE). All sections were evaluated by light microscopy and scored on a scale of 0 to 10 as described by Wang *et al*[15] (Table 2) in a blinded fashion by the same pathologist.

Serum IL-6 and TNF-α levels were analyzed with ELISA by using rat IL-6 and TNF-α kits (Invitrogen™ Rat Immunoassay Kit; Invitrogen Corporation, Carlsbad, California, USA).

Statistical analysis

The Statistical Package for Social Sciences version 11.5 (SPSS, Chicago, IL, USA) program was used for statistical analysis of data. All values were expressed as median (minimum-maximum). The Kruskal-Wallis test followed by the Mann-Whitney *U* test was used for statistical evaluation and $P < 0.05$ was accepted as the level of significance.

RESULTS

There was no significant weight difference between budesonide, levobupivacaine and sham groups; however the weight loss in the saline group was significantly higher than other groups ($P = 0.016$).

Macroscopic damage score was significantly higher in budesonide, levobupivacaine and saline groups when compared to sham group ($P = 0.001$). No significant difference was found between budesonide and levobupivacaine groups but macroscopic damage scores of these two groups were significantly lower than saline group (Budesonide *vs* Saline: $P = 0.001$; Levobupivacaine *vs* Saline: $P = 0.002$).

Various inflammatory cell accumulations and ulcerations were the common alterations observed by light microscopy in budesonide, levobupivacaine and saline groups as there was no granuloma formation or fibrosis.

Microscopic damage scores were significantly higher in budesonide, levobupivacaine and saline groups than sham group ($P = 0.014$). Although the levobupivacaine group had lower scores than the budesonide and saline groups, there was no statistical significance (Budesonide *vs* Levobupivacaine: $P = 0.436$; Saline *vs* Levobupivacaine: $P = 0.105$).

TNF-α levels did not show any significant difference among groups ($P = 0.152$). IL-6 levels in budesonide, levobupivacaine and saline groups were significantly increased when compared to sham group ($P = 0.040$). Levobupivacaine group had slightly lower IL-6 levels than budesonide and saline group, however, there was no significant difference (Levobupivacaine *vs* Saline: $P = 0.853$; Levobupivacaine *vs* Budesonide: $P = 0.247$).

These results are summarized in Table 3.

DISCUSSION

In this study, we investigated whether topical administration of levobupivacaine has an anti-inflammatory efficiency in an experimentally induced colitis model. While weight alterations and macroscopic examination scores suggested that levobupivacaine might have had anti-inflammatory effects on colitis, this suggestion was not supported by histopathological findings. Although the IL-6 levels increased secondary to induction of colitis as a proof of systemic inflammatory response, diminution of IL-6 levels after levobupivacaine administration was not significant.

Weight loss in the saline group was significantly higher when compared to levobupivacaine and budesonide or sham groups ($P = 0.016$). This might be as a result of "no treatment" as Martinsson *et al*[13] suggested. The significant weight loss in the saline group was probably due to a more serious inflammatory process than in levobupivacaine and budesonide groups. Macroscopic damage scores also supported this finding. Levobupivacaine and budesonide groups had significantly lower macroscopic damage scores than the saline group ($P = 0.001$). On the other hand, macroscopic damage scores of levobupivacaine and budesonide groups were significantly higher than the sham group ($P = 0.001$). Treatment with levobupivacaine or budesonide had equivalently improved the mucosal damage caused by colitis. Martinsson *et al*[13] suggested that ropivacaine had more protective effects than budesonide on the mucosa in a similar study. According to our findings, levobupivacaine had similar efficiency

with budesonide, which has been used in the treatment of clinical IBD[16]. Administration of levobupivacaine and budesonide diminished the inflammatory changes in our study, but this was not sufficient enough to draw back all the inflammatory process.

Microscopic damage scores did not show significant decreases in levobupivacaine and budesonide groups when compared to the saline group which suggests insufficient anti-inflammatory efficiency. However, we are not the only researchers who reported discordance between macroscopic and microscopic examination scores. Neither ropivacaine nor lidocaine administration decreased the microscopy scores even though they caused a significant decrease in macroscopic scores[13,17,18]. Maybe longer study periods are needed to examine the effects of topical local anesthetic treatment on IBD or maybe the microscopic scoring should be revisited.

TNF-α and IL-6 serum levels were measured to determine the systemic proofs of inflammatory response in our study. These cytokines act as proinflammatory factors in IBD and are usually related to severity of the disease[19]. In our study, IL-6 levels were significantly higher in levobupivacaine, budesonide and saline groups than in the sham group ($P = 0.040$). However, changes in TNF-α level did not show any significance. But it is known that TNF-α has a wide range in IBD and may sometimes not be detectable[19]. The IL-6 level in the levobupivacaine group was lower than budesonide and saline groups without statistical significance. It was reported that some local anesthetics such as lidocaine might affect the release of proinflammatory cytokines by membrane depolarization in epithelial cells[20]. The decrease of IL-6 levels in the levobupivacaine group made us think that levobupivacaine might have similar effects on cytokine levels as lidocaine. Further research might be focused on that finding.

In conclusion, despite the decrease in microscopic damage scores and IL-6 levels suggesting that topical administration of levobupivacaine might have some level of anti-inflammatory efficiency in an experimental colitis model, microscopic examination failed to support this finding. However, there may be individual differences in inflammatory response in IBD[21]. Further studies with larger groups and longer treatment periods might be beneficial in examining the potential therapeutic capacity of local anesthetics in the treatment of IBD.

COMMENTS

Background

Inflammatory bowel disease (IBD) affects many people all around the world. There are several drugs that are useful in reducing the symptoms but no definitive therapeutic agents have been defined. Local anesthetics might be useful in the treatment of IBD based on their actions that inhibit hyperactive autonomic nerves and host immune response which are key factors in the pathogenesis of IBD.

Research frontiers

Previous studies reported successful results with the local application of various local anesthetics on colonic mucosa even in comparison to conventional drugs that have been officially used in the treatment of IBD for decades.

Innovations and breakthroughs

There are various types of local anesthetics. Their action period and side effects that may be seen due to the possibility of getting into the systemic blood circulation from the areas they are applied may vary. In this study, different from the previous similar studies, the authors used levobupivacaine, which is a novel, long lasting local anesthetic with less systemic side effects.

Applications

Even though there may be some side effects if they are used in high doses or applied on very wide colonic mucosa, local anesthetics are much safer than the drugs that have been conventionally used in the treatment of IBD. Therefore, if successful results are reported with the use of local anesthetics in the treatment of IBD, a safe and cheap alternative might be defined.

Peer review

The manuscript is very interesting for the readers. In their study they explored the effect of levobupivacaine and budenosine in induced colitis. The authors analyzed macro and microscopically the resected specimen and measured the serum levels of tumor necrosis factor (TNF) and interleukin (IL)-6. It could be more interesting and support more of their results if the authors could perform immunohistochemistry detection for TNF and IL-6 in the samples.

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BRIEF ARTICLE

Stapled transanal rectal resection for obstructed defecation syndrome associated with rectocele and rectal intussusception

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Abstract

AIM: To evaluate the safety and efficacy of stapled transanal rectal resection (STARR), and to analyze the outcome of the patients 12-mo after the operation.

METHODS: From May 2007 to October 2008, 50 female patients with rectocele and/or rectal intussusception underwent STARR. The preoperative status, perioperative and postoperative complications at baseline, 3, 6 and 12-mo were assessed. Data were collected prospectively from standardized questionnaires for the assessment of constipation [constipation scoring system, Longo's obstructed defecation syndrome (ODS) score system, symptom severity score], patient satisfaction (visual analogue scale), and quality of life (Patient Assessment of Constipation-Quality of Life Questionnaire).

RESULTS: At a 12-mo follow-up, significant improvement in the constipation scoring system, ODS score system, symptom severity score, visual analog scale and quality of life ($P < 0.0001$) was observed. The symptoms of constipation improved in 90% of patients at 12 mo after surgery. The self-reported definitive outcome was excellent in 15 (30%) patients, fairly good in 8 (16%), good in 22 (44%), and poor in 5 (10%).

CONCLUSION: STARR can be performed safely without major morbidity. Moreover, the procedure seems to be effective for patients with obstructed defecation associated with symptomatic rectocele and rectal intussusception.

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Key words: Stapled transanal rectal resection; Obstructed defecation syndrome; Rectocele; Rectal intussusception

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INTRODUCTION

Obstructed defecation syndrome (ODS) is characterized by a spectrum of symptoms including difficult evacuation, excessive straining during defecation, sensation of incomplete evacuation, prolonged time to defecate, anal

pain, bleeding, and the use of external assistance to aid defecation. It has been estimated that approximately 20% of adult female population suffered from the syndrome[1,2]. The etiology of ODS is likely to be multifactorial, resulting from the interaction of functional and anatomic factors that influence the rectoanal evacuatory mechanism[3]. Furthermore, the most common clinical signs and pathophysiologic alterations associated with ODS are rectocele (RE) and rectal intussusception (RI)[4].

To date, a variety of surgical techniques including transvaginal, transperineal, transanal, and combined abdominal and vaginal approaches have been described for the treatment of ODS. However, there is no method achieving overall superiority because of different patterns of complications and high rate of recurrence. Based on the stapled hemorrhoidopexy procedure, Longo[5] proposed an alternative technique for patients with ODS caused by RE and RI called stapled transanal rectal resection (STARR). The novel technique is carried out sequentially using double circular stapler devices (PPH01, Ethicon Endo-Surgery), anteriorly and posteriorly, to restore normal rectal anatomy by strengthening rectovaginal septum and resecting redundant rectum.

STARR has been implemented rapidly and described as an effective cure for RE, RI, prolapsed hemorrhoids and even solitary rectal ulcer[6-9]. In addition, observations from several case series and multi-center trials have demonstrated a clinical benefit of the procedure in short-term follow-up[10-14]. Nevertheless, some unique and troublesome complications were also documented by a few case reports[15-18]. The current study was designed to determine the safety and efficacy of STARR in treatment of ODS related to symptomatic RE and RI, and to perform an analysis of outcomes of the patients 12 mo after surgery

MATERIALS AND METHODS

From May 2007 to October 2008, 50 female patients with ODS caused by RE and/or RI underwent STARR. Before the surgery, patients were asked about symptoms of constipation and previous histories of anorectal or gynecologic surgery. Apart from the comprehensive history, the presence and extension of any anatomical or functional abnormality were evaluated by means of a clinical examination and appropriate physiological investigations such as proctoscopy, colonoscopy, colonic transit time study, defecography and anorectal manometry. All the patients were operated on by the same surgical team, using the original techniques reported previously[6] without modifications. The protocol was approved by the ethical committee of our institution and all patients gave informed written consent.

Inclusion criteria

Patients with persistence of at least three specific symptoms of ODS (feeling of incomplete evacuation, prolonged painful straining, frequent calls to defecate, excessive toilet time, digital assistance, pelvic pain or pressure, rectal bleeding, soiling, or a feeling of prolapse). Additionally, conservative treatment with diet (1.5 L/d water, high-fiber diet), laxatives (10 g/d lactulose), enemas and/or physiotherapy had been tried in all patients without success. Moreover, there was radiologically-proven RE $\geqslant$ 3 cm, or RI < 4 cm on straining, or significant rectal barium trapping after defecation.

Exclusion criteria

Exclusion criteria were in accordance with the consensus statement published by the Pioneers group[4] and the consensus recommendations by Corman *et al*[19]. These included patients with good response to conservative treatment, slow transit constipation, enterocele, sigmoidocele, cystocele, genital prolapse, external rectal prolapse, pelvic floor dyssynergia, proctitis, active perineal infection, chronic diarrhoea, inflammatory bowel disease, neoplasia, anorectal stenosis and previous anterior resection with rectal anastomosis, and patients with anal incontinence (Cleveland Clinic Florida; Wexner Score > 7) and foreign material (such as mesh) adjacent to the rectum or with mental disorder. Furthermore, RE < 3 cm or RI $\geqslant$ 4 cm on straining was not considered straightforward candidates for the STARR procedure. Also excluded were those with general contraindications for surgery and those who declined surgical treatment.

Study design

All patients had detailed data on preoperative status, perioperative and postoperative complications. A clinical assessment was performed at baseline and at 3, 6, and 12 mo after surgery. The magnitude and degree of ODS were quantified by constipation scoring system (CSS)[20]. The validated CSS consists of eight items and the overall score ranges from 0 (normal) to 30 (severe constipation). In addition to CSS, the severity of disordered defecation was also quantified by Longo's ODS score system (Table 1) and the symptom severity (SS) score (Table 2). The summed global scores of ODS and SS range from 0-40 and 0-36, respectively, in which a higher score indicates more severe symptoms. Moreover, the index of patient satisfaction was evaluated by a visual analog scale (VAS: with a score from 0 to 10), and a higher score suggests an improvement in patient satisfaction with the surgery. Patient Assessment of Constipation-Quality of Life Questionnaire (PAC-QoL), a self-reported questionnaire, was used to measure the quality of life of patients[21,22]. The validated PAC-QoL is composed of 28 items grouped into four subscales: physical discomfort, psychosocial discomfort, worries and concerns, and satisfaction. The first three subscales were used to assess the patient dissatisfaction index, with an overall score ranging from 0 to 96 (where lower scores correspond to better quality of life). The satisfaction subscale includes four items with a global score ranging from 0 to 16, so that each patient's self-reported definitive outcome was defined as either poor (0-4), fairly good (5-8), good (9-12), or excellent (13-16)[23].

Table 1 Longo's ODS score system

ODS-score (0-40)									
Defecation frequency	1-2 def/ 1-2 d	0	2 def /wk or 3 def or attempts/d	1	1 def/wk or 4 def or attempts/d	2	< 1 def/wk or > 4 def or attempts/d		3
Straining									
Intensity	No, light	0	Moderate	1	Intensive	2			
Extension			Short time	1	Prolonged	2			
Sensation of inco-mplete evacuation	Never	0	≤ 1 time/wk	1	2 times/wk	2	> 2 times/wk		3
Recto/perineal pain/discomfort	Never	0	≤ 1 time/wk	1	2 times/wk	2	> 2 times/wk		3
Activity reduction per week	Never	0	< 25% of activity	2	25%-50% of activity	4	> 50% of activity		6
Laxatives	Never	0	< 25% of def	1	25%-50% of def	3	> 50% of def	5 Always	7
Enemas		0		1		3	5		7
Digitation		0		1		3	5		7

ODS: Obstructed defecation syndrome.

Table 2 Symptom severity score

Symptoms	None	Very short time	Some time	Most of the time	All of the time
Need laxatives/enemas	0	1	2	3	4
Unsuccessful attempts to open bowels	0	1	2	3	4
Low frequency of bowel movements	0	1	2	3	4
Pain on opening bowels	0	1	2	3	4
Bleeding on bowel opening	0	1	2	3	4
Incomplete bowel opening	0	1	2	3	4
Increased time or straining to open bowels	0	1	2	3	4
Incontinence/soiling	0	1	2	3	4
Difficulty to withstand urge to open bowels	0	1	2	3	4

Table 3 Previous anorectal or gynecologic surgery in patients undergoing STARR *n* (%)

Operation	Patients
Milligan-Morgan hemorrhoidectomy	11 (22)
Fistulectomy	1 (2)
Fistulotomy	1 (2)
Closed lateral subcutaneous sphincterotomy	1 (2)
Stapled hemorrhoidopexy	1 (2)
Episiotomy	13 (26)
Hysterectomy	9 (18)
Abdominal delivery	7 (14)

STARR: Stapled transanal rectal resection.

Surgical technique

Polyethylene glycol electrolyte solutions were given for preoperative bowel preparation, and patients received routine broad-spectrum antibiotics immediately after the induction of anesthesia. The operation was always performed under spinal anesthesia with the patient in a lithotomic position. According to recommendations for the performance of STARR[6], two circular PPH-01™ staplers (Ethicon Endo-Surgery) were used. Briefly, the anal canal was gently dilated, then the circular anal dilator (CAD33) introduced and secured with silk sutures. Three semi-circumferential purse-string sutures were positioned in the anterior rectum at approximately 1, 2, and 3 cm above the haemorrhoidal apex. The first PPH-01 stapler was inserted and the posterior rectal wall was protected with a spatula. The ends of sutures were delivered through the specific holes of the stapler, and tension was applied to prolapse the removed tissues into the stapler housing, making sure that the posterior vaginal wall had not been incorporated, the stapler was closed and fired. By the same procedure, two semi-circumferential purse-string sutures and a second PPH-01™ stapler were performed on the posterior rectal wall. Subsequent bleeding from the staple line was controlled with full-thickness 2-0 Vicryl™ stitches, and "posterior staple bridge" was divided with scissors.

Statistical analysis

Statistical analysis was performed using paired *t* test for continuous variables, and Wilcoxon's signed-rank test for quantitative variables. The total scores of CSS, ODS, SS, VAS and PAC-QoL were expressed as mean values with 95% confidence intervals (CI). A P value < 0.05 was considered statistically significant.

RESULTS

There were 50 female patients (median age, 53.7 years; range, 30-70 years). As shown in Table 3, 43 (86%) patients had experienced 1-5 vaginal deliveries, 13 (26%) had experienced at least one episiotomy, and 36 (72%) had undergone prior anorectal or gynecologic surgeries. All the patients had symptoms of outlet obstruction (Table 4). Median operating time was 28 min (range,

Table 4 Presenting symptoms and early complications in patients undergoing STARR *n* (%)

Symptoms	Incidence	Early complications	Incidence
Excessive straining	43 (86)	Defecatory urgency	21 (42)
Feeling of incomplete evacuation	42 (84)	Bleeding[3]	4 (8)
Rectal bleeding[1]	32 (64)	Incontinence to flatus	3 (6)
Rectoperineal discomfort	23 (46)	Acute urinary retention	2 (4)
Abdominal distension	21 (42)	Severe pain	2 (4)
Abdominal pain	20 (40)	Anal fissure	1 (2)
Feeling of rectal obstruction	16 (32)	Rectovaginal fistula	0
Laxatives[2]	34 (68)	Perianal sepsis	0
Enema	20 (40)	Staple line dehiscence	0
Rectal or vaginal digitation	17 (34)	Mortality	0

[1]> 1 episode/wk; [2]> 2 episodes/mo; [3]No treatment was required.

Table 5 Intermediate complications in patients undergoing STARR *n* (%)

Symptoms	3 mo	6 mo	12 mo
Defecatory urgency	5 (10)	3 (6)	1 (2)
Incontinence to flatus	1 (2)	1 (2)	1 (2)
Incontinence to feces	1 (2)	1 (2)	1 (2)
Chronic pain	2 (4)	1 (2)	1 (2)
Constipation	4 (8)	5 (10)	5 (10)
Rectal stenosis	0	0	0
Rectal diverticulum	0	0	0

20-50 min). The mean vertical height of the resected specimen was 3.8 cm (range, 2.5-4.8 cm) anteriorly and 2.0 cm (range, 2.0-4.0 cm) posteriorly; the mean horizontal length was 5.9 cm (range, 4.6-7.2 cm) anteriorly and 4.4 cm (range, 3.2-5.3 cm) posteriorly. The only intraoperative incident was subsequent bleeding from the anastomotic ring, which occurred in 92% of cases and was secured with hemostatic stitches.

Early complications are summarized in Table 4. The most common morbidity after surgery was defecatory urgency, and the incidence was 42% during the first postoperative week. Bleeding occurred in 4 (8%) patients, but the symptom was minor and no further surgical intervention was required. Other recorded complications were acute incontinence to flatus (6%), acute urinary retention (4%), severe pain (4%) and anal fissure (2%). No staple line dehiscence, massive rectal hemorrhage, rectovaginal fistula, perianal sepsis and postoperative mortality occurred.

In addition, the postoperative complications were followed up for one year. As shown in Table 5, the frequency of defecatory urgency decreased dramatically with time and was 10% at 3-mo, 6% at 6-mo and merely 2% at 12-mo follow-up. Incontinence to flatus was a problem in only 1 (2%) elderly patient at a 3-mo follow-up. Moreover, the patient developed incontinence and remained unchanged during a 12-mo follow up. Another 2 (4%) cases had chronic pain during 3 mo after resection, whereas one resolved at a 6-mo follow-up. Forty-six (92%) patients had a clinical benefit at 3 mo, however, constipation recurred in one patient at 6 mo after the STARR procedure. At a follow-up of 12 mo, the symptoms of constipation improved in 45 (90%) patients. No rectal stenosis and rectal diverticulum occurred.

The variation of the CSS, ODS, SS and VAS scores are presented in Figure 1A-D. Overall, a significant reduction in CSS, ODS and SS scores was observed at 12 mo as compared with baseline [CSS score at baseline *vs* 12 mo: 15.58 (95% CI: 14.40-16.76) *vs* 5.68 (95% CI: 4.34-7.02); ODS score: 17.54 (95% CI: 16.13-18.95) *vs* 5.92 (95% CI: 4.31-7.53); SS score: 15.22 (95% CI: 13.91-16.53) *vs* 4.52 (95% CI: 3.29-5.75); $P < 0.0001$]. The differences indicated that patients undergoing STARR had an improvement in the symptoms of obstructed defecation. Additionally, the VAS score at 12 mo was more markedly increased than that at baseline [VAS score at baseline *vs* 12 mo: 4.07 (95% CI: 3.79-4.35) *vs* 7.59 (95% CI: 7.10-8.08), $P < 0.0001$]. The results were consistent with the improvement in CSS, ODS and SS scores, and suggested that patients undergoing STARR also had an obvious improvement of patient satisfaction. Summarized data from the individuals showed that the PAC-QoL score at 12 mo was significantly lower than that at baseline [PAC-QoL (dissatisfaction index) at baseline *vs* 12 mo: 47.78 (95% CI: 44.25-51.31) *vs* 8.14 (95% CI: 6.13-10.15), $P < 0.0001$]. Furthermore, the self-reported definitive outcome was excellent in 15 patients (30%), fairly good in 8 (16%), good in 22 (44%), and poor in 5 (10%). These observations demonstrated that most of the patients (90%) undergoing STARR had noticeable improvement in both the quality of life and the index of satisfaction.

DISCUSSION

ODS is an emerging and challenging clinical problem, the pathophysiology of which remains to be clearly defined. RE and RI, however, are the two most frequent anatomic defects associated with ODS. Although various surgical procedures have been described for the treatment of the syndrome, many of these are unsuitable for patients accompanied with RE and RI[24]. Notably, STARR has been demonstrated as an alternative operation and a relatively noninvasive surgical technique for ODS caused by RE and RI. The novel procedure aims to correct rectocele, resect internal prolapse, restore anatomy, correct rectal volume, and improve function[10]. Nevertheless, limited evidence has been available about the safety and short-term efficacy of STARR so far[23,25,26].

In our experience, STARR can be performed safely without major morbidity. The only intraoperative incident was subsequent bleeding from the staple line which happened in 92% of patients. Given that a full thickness segment of rectal wall was excised by the STARR staplers, the anastomotic ring should be meticulously checked and carefully secured with stitches whenever necessary. The current dada documented 4 (8%) cases with mild bleeding from the staple line postoperatively,

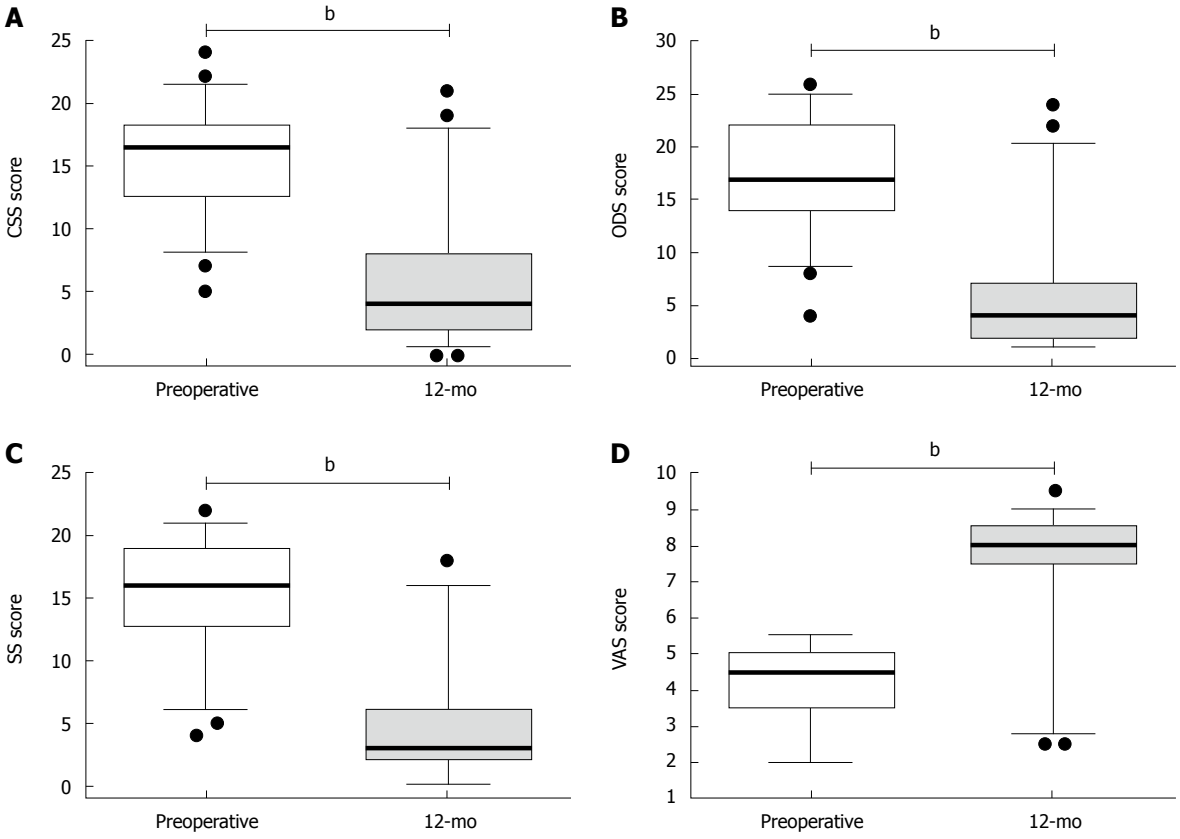


Figure 1 Comparison of scores at baseline and at a 12-mo follow-up. A: Comparison of constipation scoring system (CSS) scores; B: Comparison of Longo's obstructed defecation syndrome (ODS) scores; C: Comparison of symptom severity (SS) scores; D: Comparison of visual analog scale (VAS) scores. A significant reduction in the CSS, ODS, SS and VAS scores was observed at 12 mo after surgery as compared with baseline (paired *t* test , $^{b}P < 0.0001$).

but no one required further surgical intervention. Additionally, no severe complications associated with bleeding such as massive rectal hemorrhage and secondary anastomotic sepsis occurred in this study. Furthermore, no patient had recto-vaginal fistula. It may be because to a large extent, that much attention was paid to avoiding any entrapment of the posterior wall of vagina in the process of operation. Even though a few worrisome complications[15-18] including staple line dehiscence, rectal diverticulum, pelvic infection and even fulminating necrotizing pelvic fasciitis occurred following the STARR procedure in some studies, none of our patients developed any of the complications.

Accumulating evidences have shown that defecatory urgency was the most common complaint in the immediate and intermediate recovery periods after STARR[26,27]. Consistently, our data showed that the incidence was 42% during the first postoperative week and 10% at 3-mo, 6% at 6-mo and merely 2% at a 12-mo follow-up. The frequency was decreased dramatically with time, indicating that the complication was largely self-limited. Contrary to Titu's observations[27], the complaint was not present with an obviously regular rhythm and symptom-relieving period character in our patients. Although the exact etiopathogenesis of defecatory urgency is unclear, it may reflect the inflammatory response related to the staple line, presence of irritable rectum, and reduced rectal capacity or compliance. The incidence of incontinence to flatus was 6% during the first week after surgery, but was only 2% at a 3-mo follow-up. The marked improvement manifested that it may be a procedure-related complication caused by transient sphincteric impairment during instrumentation. Nevertheless, one elderly patient with postoperative defecatory urgency was concomitant with incontinence during a 12-mo follow-up. The potential relationship between defecatory urgency and incontinence need to be further elucidated.

Even though there is no acknowledged and standardized assessment to quantitate ODS, a dedicated score represents an essential tool, which can be used for the clinical staging of ODS for subsequent surgery and for the evaluation of therapeutic results[28]. Therefore, the validated CSS, Longo's ODS score system, SS score, VAS, and the validated PAC-QoL were used for clinical assessment in this study. Compared with the preoperative results, an evident reduction in the CSS, ODS and SS scores was exhibited at 12 mo after surgery. The variation demonstrated the efficacy of STARR in relieving symptoms of obstructed defecation. Furthermore, the significant differences between preoperative and postoperative mean total scores of VAS and PAC-QoL indicated an improvement in both the patient satisfaction index and the quality of life. At a 12-mo follow-up, it was noteworthy that the self-reported definitive outcome was excellent in 15 patients (30%), fairly

good in 8 (16%), good in 22 (44%), and poor in 5 (10%). The symptoms of constipation improved in 45 (90%) patients. However, a few studies have reported the limited effect of STARR for the treatment of ODS[15,16]. The different inclusion/exclusion criteria, the limited expertise of STARR surgery and the absence of routine surgical practice may account for the discrepant results. In addition, the long-term benefits warrant further investigations.

Intriguingly, the Contour Transtar stapler™ (Ethicon Endo-Surgery, Inc.) device has been introduced more recently as a modification of the STARR procedure[29,30]. Transtar has the potential benefit of being able to tailor the amount of rectal wall to be resected. Meurette *et al*[31] suggested that it would be wise to select the STARR procedure for a predominant "isolated" RE and the Transtar procedure for a high grade RI. Therefore, further research into this area is required to optimize patient selection, and the difference in function and efficacy between STARR and Transtar remains to be observed.

COMMENTS

Background

Obstructed defecation syndrome (ODS) is one of the most widespread clinical problems which frequently affect middle-aged females. Rectocoele (RE) and rectal intussusception (RI) are the two most common clinical signs and anatomic defects associated with ODS. Stapled transanal rectal resection (STARR) is a new procedure for obstructed defecation due to RE and RI.

Research frontiers

Previous studies have shown a clinical benefit of the STARR procedure for ODS related to RE and RI in short-term follow-up. However, limited effect and some serious complications were also described. In this study, the authors demonstrated that STARR seems to be safe and effective for selected patients presenting with ODS caused by RE and RI.

Innovations and breakthroughs

This is an original study taking place in a single centre that describes the safety and feasibility of STARR for the management of ODS due to symptomatic RE and RI. The study confirms that this technique can be performed safely without major morbidity. Furthermore, the symptoms of constipation relieved in 90% of patients with significant improvement in both the quality of life and the index of satisfaction at 12 mo after surgery.

Applications

Although the current study indicates a short-term clinical benefit, a longer follow-up should be made to establish the true value of STARR. Moreover, further studies are needed to optimize patient selection and compare the efficacy of this technique with that of other surgical procedures for patients with ODS.

Terminology

STARR is a relatively noninvasive surgical technique using double circular stapler devices to resect a full thickness segment of rectal wall. The novel procedure has been utilized for the treatment of RE, RI, prolapsed hemorrhoids, solitary rectal ulcer and even rectal tumor.

Peer review

This work is well planned and structured. The authors report good short-term results with the STARR procedure for obstructed defecation due to a RE or RI.

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BRIEF ARTICLE

Xba I polymorphisms of apolipoprotein B gene: Another risk factor of gallstone formation after radical gastrectomy

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Abstract

AIM: To prospectively investigate the association between the *Xba* I polymorphisms of apolipoprotein B (*APOB*) gene and gallstone formation following gastrectomy.

METHODS: The study was conducted between January 2005 and December 2006. A total of 186 gastric cancer patients who had undergone radical gastrectomy were grouped according to *Xba* I polymorphisms of *APOB* gene (X^+X^- group, n = 24 and X^-X^- group, n = 162) and compared. The *Xba* I polymorphisms of *APOB* gene were detected by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).

RESULTS: The incidence of gallstone was significantly higher in the X^+X^- group than in the X^-X^- group [54.2% *vs* 9.3%, RR = 5.85 (2.23-15.32), P < 0.001]. The serum levels of total cholesterol (TC) and low-density lipoprotein (LDL) were higher in the X^+X^- than in the X^-X^- group (4.02 ± 1.12 *vs* 3.48 ± 0.88, P = 0.004 before surgery and 3.88 ± 1.09 *vs* 3.40 ± 0.86, P = 0.008 after surgery). LDL was 2.21 ± 0.96 *vs* 1.89 ± 0.84 (P = 0.042) before surgery and 2.09 ± 0.95 *vs* 1.72 ± 0.85 (P = 0.029) after surgery in the two groups. No relationship was found between *Xba* I polymorphisms and gallbladder motility.

CONCLUSION: In Chinese patients after radical gastrectomy, X^+ allele of *APOB* gene is another risk factor for the development of gallstone besides the gallbladder motility disorder after surgery.

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Key words: Gastric cancer; Gastrectomy; Gallstone; Apolipoprotein B gene; Polymorphism

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INTRODUCTION

The incidence of gallstones is higher in patients after radical gastrectomy than in the general population[1]. The current literature suggests that this higher incidence is related to gallbladder motility disorder after surgery resulting from severance of the vagus nerve, non-physiological reconstruction of the gastrointestinal tract and lymph node dissection[2-5]. However, it is also observed in clinical practice that following the same type of surgery on patients with the same stage of gastric cancer, some of them

do and the others do not develop gallstones. This suggests that besides gallbladder dysmotility, there are some possible inherent factors of gallstone formation after gastrectomy. In recent years, several studies have reported that polymorphisms in the apolipoprotein B (*APOB*) gene were associated with gallstone diseases[6,7]. Apolipoprotein B is a key protein in lipid metabolism. It plays an important role in the homeostasis of low-density lipoprotein (LDL) cholesterol in plasma serving as a ligand for receptor-mediated endocytosis of LDL. Several polymorphic forms of *APOB* alleles have been reported to be associated with disorders like coronary heart disease and non-insulin dependent diabetes mellitus[8]. The *APOB-Xba* I polymorphism has been found to be associated with increased serum lipids. Genetic polymorphisms in the *APOB* gene have also been reported to be associated with susceptibility to cholesterol gallstones[9]. However, the effects of *APOB-Xba* I gene polymorphism in patients who had undergone radical gastrectomy for gastric cancer without any previous history of cholelithiasis, abnormal gallbladders motility, diabetes, hyperlipidemia, hyperparathyroidism and other metabolic disorders is unknown. The present study prospectively investigated the association between the *Xba* I polymorphisms of *APOB* gene and gallstone formation after radical gastrectomy.

MATERIALS AND METHODS

Patients

The study enrolled 206 patients who underwent radical gastrectomy for gastric cancer in Zhongshan Hospital between January 2005 and December 2006. The inclusion criteria included age less than 60 years; biopsy proven gastric cancer; normal gallbladder motility; no gallstone or other hepatobiliary system diseases, no metabolic disorders and important organs dysfunction. To avoid biases, all patients were operated upon by the same surgical team with D2 dissection. Complete data were available for only 186 patients as 20 patients were lost to follow-up (3 patients in X^+X^- group and 17 patients in X^-X^- group). These patients were grouped according to *Xba* I polymorphisms of *APOB* gene. For analyzing the effects of *Xba* I polymorphisms, blood was also collected before and 6 mo after gastrectomy. The serum lipids, lipoproteins and apolipoproteins were determined. The levels of total cholesterol (TC), LDL and *APOB* were tested by biochemical autoanalyser (HITACHI7600). The gallbladder motility was examined by detecting gallbladder emptying fraction (GBEF) using nuclide imaging before and 6 mo after gastrectomy. Gallstone formation was determined by periodic ultrasonography after gastrectomy.

Informed consent was obtained from all patients, and the study was approved by the ethics committee of the hospital.

Determination of DNA polymorphism

Leukocyte genomic DNA was extracted from 5 mL of peripheral blood by TianGen Genetic DNA Kit (TianGen Biotech Co., Ltd.). The desired segments were amplified by PCR using the APOB *Xba* I protocols with the primers: 5'(5'GGAGACTATTCAGAAGCTAA3') and 3'(3'GAAGAGCCTGAAGACTGACT5'). The final amplification products were submitted to digestion with the restriction enzymes (*Xba* I) and the variations were visualized after electrophoresis on 1.5% agarose gel with ethidium bromide under ultra-violet light, followed by photographic documentation. PCR cycle for amplification of *APOB* gene was performed at 95℃ for 5 min followed by 95℃ for 45 s, annealing at 58℃ for 45 s and extension at 72℃ for 45 s in 33 cycles. A final extension was conducted for 10 min at 72℃. The products were digested by *Xba* I restriction enzyme at 37℃ for 12 h.

Statistical analysis

Data are expressed as the means ± SD. Means of age, body mass index (BMI), TC, LDL and *APOB* in different groups were compared using Student's t test. Gender, stage, types of gastrectomy and reconstruction were compared using χ^2 test. The differences between each genotype of *Xba* I polymorphisms and the incidence of gallstones were assessed by Fisher's exact test. Interaction between *Xba* I polymorphisms and gallbladder motility in the development of gallstone after gastrectomy was estimated by logistic regression analysis. SPSS statistical software, version 15.0 for Windows was used for all analyses. $P < 0.05$ was considered statistically significant.

RESULTS

Demographic data of X^+X^- group and X^-X^- group

The frequencies of X^+X^- and X^-X^- in 186 patients were 24 (13%) and 162 (87%), respectively. No genotype with X^+X^+ was detected in the study. Demographic data of X^+X^- group and X^-X^- groups are shown in Table 1. The median follow-up time was 28 mo (range: 24-32 mo). The mean age and BMI at the time of gastrectomy was 56.1 ± 8.2 years (range: 38-80 years) and 21.9 ± 1.8 kg/m^2 (range: 19.2-25.1 kg/m^2), respectively. With regard to type of gastrectomy, 7 (4%), 162 (87%), and 17 (9%) patients underwent a proximal, distal and total gastrectomy, respectively. Age and BMI did not differ significantly between the X^+X^- and X^-X^- groups. With respect to TMN stage, gallbladders motility after surgery, types of gastrectomy and reconstruction, there were no significant differences between the two groups (Table 1).

Gallstone formation after radical gastrectomy in X^+X^- and X^-X^- groups

Of the 186 patients, 28 (15.1%; 13 in X^+X^- group, 15 in X^-X^- group) developed gallstones with in a mean follow-up period of 28.4 ± 2.7 mo. The incidence of gallstone formation was significantly higher in the X^+X^- group than in the X^-X^- group (54.2% *vs* 9.3%, relative risk 5.85, 95% CI: 2.23-5.32, $P < 0.001$). The time period from gastrectomy to detection of the gallstones ranged from 6 to 32 mo with a median of 20 mo. The timing of gallstones formation showed no difference between the two groups

Table 1 Demographic data of 186 gastric cancer patients who underwent gastrectomy with D2 dissection in the X^+X^- and X^-X^- groups (mean ± SD) *n* (%)

	X^+X^- group (*n* = 24)	X^-X^- group (*n* = 162)	*P* value
Age (yr)	52.4 ± 5.1	56.8 ± 7.4	0.998
Gender			
Male	16 (66.7)	110 (67.9)	0.904
Female	8 (33.3)	52 (32.1)	
BMI (kg/m^2)	22.0 ± 1.2	21.7 ± 1.6	0.379
TNM stage[1]			
Ⅰ A	1 (4.2)	7 (4.3)	0.996
Ⅰ B	4 (16.7)	31 (19.1)	
Ⅱ	8 (33.3)	52 (32.1)	
Ⅲ A	8 (33.3)	49 (30.3)	
Ⅲ B	3 (12.5)	23 (14.2)	
Type of gastrectomy			
Proximal gastrectomy	1 (4.2)	6 (3.7)	0.820
Distal gastrectomy	20 (83.3)	142 (87.7)	
Total gastrectomy	3 (12.5)	14 (8.6)	
Type of reconstruction			
Without duodenal exclusion	14 (58.3)	96 (59.3)	0.991
With duodenal exclusion	10 (41.7)	66 (40.7)	
Gallbladders motility after surgery			
Normal	10 (41.7)	62 (38.3)	0.750
Abnormal	14 (58.3)	100 (61.7)	

[1]According to the Classification of IUCC. BMI: Body mass index.

Table 2 Gallstone formation after radical gastrectomy

	X^+X^- group (*n* = 13)	X^-X^- group (*n* = 15)	*P* value
Incidence of gallstone	54.2% (13/24)	9.3% (15/162)	< 0.001
Time period from gastrectomy to detection of the gallstones (mo)	16.4 ± 8.4	21.7 ± 7.4	0.085
Gallbladders motility			
GBEF before surgery	50.9% ± 12.5%	51.3% ± 10.3%	0.863
GBEF after surgery	30.3% ± 20.1%	29.6% ± 19.1%	0.868
Follow-up period after gastrectomy (mo)	28.3 ± 1.1	28.6 ± 1.2	0.249

n: Number of patients with gallstone; GBEF: Gallbladder emptying fraction.

(Table 2). For further analysis, whether interaction existed between *Xba* I polymorphisms and gallbladder motility in the development of gallstone after gastrectomy was estimated by logistic regression. As a result, no interaction effect of *Xba* I polymorphisms was found with gallbladder motility (Table 3). To analyze the higher incidence of gallstone in X^+X^- group, the serum concentrations of TC, LDL and APOB were compared between the two groups (Table 4). The serum levels of TC and LDL were significantly higher in X^+X^- group than in X^-X^- group both before and after surgery, but there was no statistical difference in APOB values between the two groups.

DISCUSSION

Formation of gallstones after radical gastrectomy is the result of a very complex interaction of various factors.

Table 3 Association of *Xba* I genotypes with gallstone after gastrectomy, stratified by gallbladder motility status

	Normal gallbladder motility[1]		Abnormal gallbladder motility[1]		Pinteraction
	Gallstone (+)	Gallstone (-)	Gallstone (+)	Gallstone (-)	
X^+X^- group	3	7	10	4	0.701
X^-X^- group	1	61	14	86	

[1]According to the GBEF (gallbladder emptying fraction), estimated by logistic regression analysis.

Table 4 Serum levels of TC, LDL and APOB in X^+X^- and X^-X^- groups (mean ± SD)

	X^+X^- group (*n* = 24)	X^-X^- group (*n* = 162)	*P* value
Total cholesterol (mmol/L)			
Before surgery	4.02 ± 1.12	3.48 ± 0.88	0.004
After surgery	3.88 ± 1.09	3.40 ± 0.86	0.008
Low-density lipoprotein (mmol/L)			
Before surgery	2.21 ± 0.96	1.89 ± 0.84	0.042
After surgery	2.09 ± 0.95	1.72 ± 0.85	0.029
Apolipoprotein B (μg/L)			
Before surgery	0.73 ± 0.15	0.70 ± 0.12	0.142
After surgery	0.72 ± 0.14	0.68 ± 0.12	0.072

TC: Total cholesterol; LDL: Low-density lipoprotein; APOB: Apolipoprotein B.

Destruction of neural structures, such as the vagal nerves, represents one well-known risk factor[2,10,11]. It is speculated that complete amputation of the vagal trunk with dissection of the esophagus (as in total gastrectomy) has a great influence on the contractile ability of the gallbladder. A previous experimental study showed that gastrectomy abolishes phasic contraction of the gallbladder, resulting in an absence of agitation of the gallbladder bile and mixing of gallbladder bile with fresh hepatic bile, increasing the propensity for salt precipitation and gallstone formation[12]. The type of reconstruction is also closely related to gallstone formation because the passage of food through the duodenum stimulates a variety of hormonal secretions such as cholecystokinin[13,14] and this hormone causes contraction of the gallbladder through the hormonal regulation system. It is postulated that exclusion of the duodenum leads to changes in the pattern of cholecystokinin secretion, resulting in decreased gallbladder contraction and an increased risk of gallstone formation[15].

Almost all previous studies have attributed gallstone formation to gallbladder motility disorder after radical gastrectomy. However, it is known from clinical experience that after the same type of surgery is performed on patients with the same stage of gastric cancer, some of them do and the others do not develop gallstones. In the present study, of 114 patients with abnormal gallbladder motility after surgery, 24 patients (21%) developed gallstones and the others did not. It suggests that the theory

of gallbladder motility disorder cannot completely explain gallstone formation after radical gastrectomy. The present study showed that the incidence of gallstone formation was significantly higher in the X^+X^- group than in the X^-X^- group (54.2% *vs* 9.3%), RR 5.85 (2.23-15.32), suggesting that the X^+X^- genotype may be another risk factor in gallstone formation after radical gastrectomy besides gallbladder motility disorder. Furthermore, we found that X^+X^- genotype had no interaction effect with gallbladder motility.

The *Xba* I RFLP-PCR in exon 26 of the *APOB* gene involves the 2488th nucleotide (ACC→ACT). The presence of thymine creates a restriction site for the *Xba* I enzyme characterizing the X^+ allele, whereas its absence determines the X^- allele. These are synonymous variations and so they do not affect the amino acid sequence of *APOB*[16,17]. Hegele *et al*[18] suggested that the allelic frequencies varied between races or within genetic subgroups of a single race. Caucasians have a much higher frequency of X^+ allele than Chinese. In our study, the frequency of X^+ allele in Chinese population was 13% similar to other regional reports and much lower than that reported in Caucasians, South Asians and Brazilians[8,19-21]. Law *et al*[22] and Rajput-Williams *et al*[23] also found a positive association between the X^+ allele and cholesterol concentration, whereas others did not demonstrate such correlation[24,25]. In the present study, we observed that in Chinese population, the serum levels of TC and LDL were significantly higher in X^+X^- group than in X^-X^- group both before and after gastrectomy, but no statistical difference was found in APOB values between the two groups. Since the X^+ and X^- variations are synonymous, the APOB concentration showed no difference between X^+X^- and X^-X^- groups. It is possible that the X^+ allele is in linkage disequilibrium with an unknown variation in the *APOB* gene or with a variation in another gene that influences the levels of TC and LDL, resulting in an increased saturation of bile cholesterol, leading to the gallstone formation[26,27].

In conclusion, the X^+ allele of *APOB* gene in Chinese patients is another important risk factor of gallstone formation after radical gastrectomy. Since this is the first report in a relatively small cohort, the findings should be validated in more independent studies.

COMMENTS

Background

The cause of higher incidence of gallstone after radical gastrectomy is very complex. Besides the gallbladder motility disorder, *Xba* I polymorphisms of apolipoprotein B (*APOB*) gene may be associated with gallstone development.

Research frontiers

APOB is a key protein in lipid metabolism. However, the effects of *APOB-Xba* I gene polymorphism on patients who underwent radical gastrectomy for gastric cancer are unknown. In this study, the authors demonstrate that X^+ allele of *APOB* gene is an important risk factor of gallstone formation after gastrectomy.

Innovations and breakthroughs

Previous studies have attributed higher incidence of gallstone after radical gastrectomy to gallbladder motility disorder. This is the first study to report the relationship between genetic factor (*APOB-Xba* I gene polymorphism) and gallstone formation in gastrectomized patients.

Applications

It is important to screen the high-risk group of gallstone in patients who underwent radical gastrectomy in order to prevent the gallstone formation after surgery.

Peer review

This is a small but interesting study with a reasonable rationale - examining whether a suspected genetic risk factor for gallstones increases the risk of gallstones after gastrectomy (which is a known major risk factor for gallstones). The authors indicated X allele of *APOB* gene is another risk factor for development of gallstone formation after radical gastrectomy. This present data is clear and interesting.

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BRIEF ARTICLE

Clinicopathologic significance of slug expression in human intrahepatic cholangiocarcinoma

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Abstract

AIM: To explore the expression and function of slug, a transcriptional repressor, in human intrahepatic cholangiocarcinoma (IHCC) and identify its role in IHCC progression.

METHODS: Expression of slug was detected in 36 cases of IHCC and 12 cases of normal intrahepatic bile ducts and liver parenchyma by immunohistochemistry. The patients were divided into low slug expression group (< 20% of carcinoma cells stained) and high slug expression group (≥ 20% of carcinoma cells stained). Slug expression was correlated with clinicopathological parameters of IHCC patients. The patients were defined as short-term survivors if their survival time was < 12 mo and as long-term survivors if their survival time was ≥ 12 mo.

RESULTS: Slug was not expressed in normal liver epithelium samples, lowly expressed in 15 tissue samples (10 -, 5 +) and highly expressed in 21 tissue samples (16 ++; 5 +++) from IHCC patients. The survival rate of patients with a low slug expression was 33.3% (n = 5) and 66.7% (n = 10), respectively. The survival rate of patients with a high slug expression was 61.9% (n = 13) and 38.1% (n = 8), respectively (P = 0.02). Lymph node metastasis was found in 4 (26.7%) out of the 15 patients with a low slug expression and in 14 (66.7%) out of the 21 patients with a high slug expression, respectively. The incidence rate of lymph node metastasis increased with the increasing slug expression level (P = 0.003), and higher in patients with a high slug expression than in those with a low slug expression. Slug expression did not significantly correlate with the tumor size and stage or histologic grade, or with the gender and age of patients.

CONCLUSION: Slug expression is a novel prognostic marker for IHCC with lymph node metastasis.

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Key words: Intrahepatic cholangiocarcinoma; Transcriptional repressor Slug; Clinicopathologic significance

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INTRODUCTION

Intrahepatic cholangiocarcinoma (IHCC) is the second

most common liver cancer in adults, accounting for approximately 10% of all primary liver cancers[1,2]. Complete surgical resection is the only procedure for IHCC. The prognosis of IHCC is very poor because of its advanced stage when diagnosed and early metastasis. The prognostic factors for IHCC include curative resection, hepatitis B surface antigen (HBsAg), as well as tumor, node and metastasis (TNM) stage, tumor size, lymph node metastasis, perineural invasion, portal vein invasion, and intrahepatic metastasis[3-7].

Slug, a member of the snail family of transcription factors, plays a crucial role in regulation of epithelial-mesenchymal transition by suppressing several epithelial markers and adhesion molecules, including E-cadherin[8-12]. It has been shown that slug is overexpressed in breast, esophageal squamous cells, colorectal, gastric carcinomas and lung adenocarcinoma[13-17], accompanying increased lymph node metastasis. In this study, we investigated whether slug expression is correlated to the invasion and lymph node metastasis of IHCC and whether it is a significant prognostic factor after surgical resection.

MATERIALS AND METHODS

Patients and tissue samples

From October 1998 to December 2008, 36 IHCC patients (21 men and 15 women) at the age of 31-76 years (mean: 60 ± 9 years) underwent liver resection at the Second Department of General Surgery in Affiliated Hospital of Qingdao University (China). All the patients were histologically confirmed to have IHCC. Thirty-six patients underwent hepatectomy with lymph node dissection in 30, and common bile duct resection with biliary reconstruction in 18. Lymph node metastasis, lymphovascular invasion, and distant metastasis were detected in 18, 12, and 8 patients, respectively. Clinicopathologic parameters were recorded and compared.

Histologic examination

Resected specimens were cut along the largest diameter, fixed with formalin and embedded in paraffin. The sections were stained with hematoxylin and eosin (HE). Tumor diameter, bile duct spread type, vascular and lymphatic duct permeation of cancer cells, perineural invasion of cancer cells, and lymph node metastasis were observed and recorded.

Immunohistochemistry

Mouse monoclonal anti-slug antibodies diluted at 1:100 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and secondary antibodies to rabbit anti-mouse IgG (Bost, Wuhan, China) were used for immunostaining. Formalin-fixed and paraffin-embedded tissue blocks were cut into 4-μm thick sections. After deparaffinization and blocking of endogenous peroxidase with 0.3% hydrogen peroxide in methanol for 30 min, the sections were immersed in 10 mmol/L citrate buffer (pH 6.0), dried in a microwave oven for 25 min, and washed with 50 mmol/L Tris-buffered saline (TBS; pH 7.6). After preincubation with normal goat serum (DAKO, Hamburg, Germany), the sections were incubated with appropriate primary antibodies for 1 h at room temperature, washed with TBS, incubated with secondary antibodies (diluted at 1: 50) for 30 min, and washed again with TBS. Peroxidase reaction was visualized using 0.05% diaminobenzidine tetrahydrochloride (DAB) containing 0.01% hydrogen peroxide. The sections were counterstained with hematoxylin. IHC results were assessed with a scoring system as previously described[18].

Immunohistochemical staining was expressed as percentage of positively stained carcinoma cells. Slug expression in cytoplasm of cells was evaluated. The percentage of positively stained carcinoma cells was graded as -: < 5% of carcinoma cells stained, +: ≥ 5% - < 20% of carcinoma cells stained, ++: ≥ 20% - <50% of carcinoma cells stained, and +++: ≥ 50% of carcinoma cells stained. Additionally, the examined cases were divided into low slug expression group and high slug expression group for statistical analysis.

Statistical analysis

All statistical analyses were carried out using the SPSS 11.0 software (SPSS, Chicago, USA). Differences between slug expression in tumor and its adjacent nontumorous tissues were analyzed using paired Student's *t* test. Spearman bivariate correlation was used to determine positive or negative correlations between slug protein expression and clinicopathologic parameter of IHCC patients. Relation between slug protein expression and clinicopathologic parameters of IHCC patients was analyzed using the Pearson χ^2 test. $P < 0.05$ was considered statistically significant.

RESULTS

Histologic observation

Slug was detected in 26 of 36 tumor tissue samples (72.2%). Weak staining (+), moderate staining (++), and strong staining (+++) were found in 5, 16, and 5 tumor tissue samples, respectively. Slug was lowly expressed in 15 (- and +) tumor tissue samples and highly expressed in 26 (++ and +++) tumor tissue samples, respectively. The positive slug staining (≥ 5% of carcinoma cells stained) was observed in cytoplasm of carcinoma cells. Slug was not expressed in normal intrahepatic bile ducts or liver parenchyma of 12 normal epithelium tissue samples.

Relation between slug expression and clinicopathologic features

The relation between slug expression and clinicopathologic parameters of IHCC patients is summarized in Table 1. The percentages of distant metastasis, positive nodal metastasis, and positive lymphatic invasion were significantly higher in patients with a high slug expression than in those with a low slug expression, as were the percentages of recurrence in hematogenous and lymph nodes.

The expression rate of slug was 66.7% and 33.3% in patients with a long survival time (> 12 mo) and a short

Table 1 Relation between slug expression and clinicopathologic parameters

	Slug		*P* value
	Low expression (*n* = 15)	High expression (*n* = 21)	
Gender			
Male (*n* = 21)	9	12	
Female (*n* = 15)	6	9	> 0.050
Age (yr)			
< 60 (*n* = 17)	8	9	
⩾ 60 (*n* = 19)	7	12	> 0.050
Stage			
Ⅰ-Ⅱ (*n* = 21)	6	15	
Ⅲ-Ⅳ (*n* = 15)	9	6	> 0.050
Size			
T1-2 (*n* = 21)	8	13	
T3-4 (*n* = 15)	7	8	> 0.050
Histologic grading			
G1 (*n* = 19)	10	9	
G2 + G3 (*n* = 17)	5	12	> 0.050
Lymph node metastasis			
Positive (*n* = 18)	4	14	
Negative (*n* = 18)	11	7	< 0.005
Lymphovascular invasion			
Positive (*n* = 12)	3	9	
Negative (*n* = 24)	12	12	< 0.005
Distant metastasis			
Positive (*n* = 8)	1	7	
Negative (*n* = 28)	14	14	< 0.001
Hematogenous recurrence			
Positive (*n* = 12)	3	9	
Negative (*n* = 24)	12	12	< 0.010
Lymph node recurrence			
Positive (*n* = 8)	2	6	
Negative (*n* = 29)	13	16	< 0.005
Survival time (yr)			
< 1	5	13	
⩾ 1	10	8	< 0.050

survival time (< 12 mo), respectively. A high slug expression was seen in 38.1% and 61.9% of long-term survivors and short-term survivors, respectively ($P = 0.04$, Table 1).

DISCUSSION

Slug (Snai2), a snail-related zinc-finger transcription factor, is critical for the normal development of neural crest-derived cells and loss-of-function. It has been shown that slug mutations contribute to pie baldism and Waardenburg syndrome type 2 in a dose-dependent manner and slug is aberrantly expressed in a number of tumors, thus contributing to tumorigenesis[19-22]. It has been reported that overexpression of slug plays a role in progression of many malignancies such as breast, colon, and gastric cancers[13,15,16]. Slug up-regulation correlates with deeper invasion, lymph node metastases, and stage of esophageal squamous cell carcinoma[14]. It was reported that slug up-regulation is significantly related with distant metastases and advanced pTNM stage of gastric carcinoma[16]. In this study, however, we were unable to find a significant association between slug up-regulation and lymph node metastases of IHCC.

In this study, the expression of slug protein was detected by immunohistochemistry in 26 (72.2%) out of 36 IHCC tissue samples, indicating that slug is expressed in IHCC. However, slug was not expressed in normal intrahepatic bile ducts or liver parenchyma.

The poor prognosis of IHCC patients with a high slug expression seemed to be related to the high incidence of lymph node metastases. Slug was closely correlated with lymph node invasion, lymphovascular invasion, and distant metastasis ($P < 0.005$). The patients with lymphatic invasion and distant metastasis had a high slug expression. Furthermore, the higher recurrence of lymph node metastasis in patients with a high slug expression also indicated that slug might play an important role in lymph node metastasis of IHCC. In various other cancers, such as breast cancer[13], and esophageal squamous cell carcinoma[14], slug is positively correlated with lymph node metastasis, also indicating that slug may play an important role in invasion of sinusoidal space and portal tract. Nevertheless, the precise mechanism underlying lymphatic duct invasion and lymph node metastasis has yet to be clarified. Another mechanism underlying the affinity of cancer cells for lymphatic tissue may also present. Therefore, it is necessary to determine the precise mechanism underlying lymph node metastasis of IHCC.

In this study, high slug expression was significantly related to a short survival time of IHCC patients and was an independent factor for poor prognosis of IHCC patients. The outcome after surgery was significantly poorer in patients with a high slug expression than in those with a low slug expression, suggesting that slug may be involved in the progression of IHCC and serve as a marker for predicting the malignancy of IHCC after surgery.

It has been shown that slug is expressed in several malignancies, such as breast and gastric cancer[13,16]. Slug expression is independently correlated with tumor invasiveness in breast cancer[13]. It has also been shown that slug is present in all infiltrating ductal carcinomas with lymph node metastases[16] and slug expression is inversely correlated with the differentiation of breast cancer[13]. In our experiment, no correlation was found between slug expression and tumor differentiation, which may be attributed to the limited number of cases.

In conclusion, slug is expressed in IHCC and is an independent indicator of poor prognosis. Slug can be sued as a marker for predicting the outcome of patients with IHCC after surgical resection. IHCC patients with positive slug expression should be followed up carefully.

COMMENTS

Background

The incidence of intrahepatic cholangiocarcinoma (IHCC) has been increasing in recent years. However, its cause remains largely unknown. Prognosis of IHCC is very poor because of early metastasis. Several recent studies have shown that slug is overexpressed in many cancers accompanying lymph node metastasis, which is related to shorter survival time of IHCC patients.

Research frontiers

In this study, slug was overexpressed, which is related to increased lymph node metastasis and shorter survival time of IHCC patients.

Innovations and breakthroughs

Recent reports have highlighted the importance of slug in carcinogenesis. Slug

is overexpressed in many cancers including IHCC, which is related to increased lymph node metastasis and shorter survival time of IHCC patients.

Applications

This study may foster a future strategy against IHCC by elucidating whether slug expression is correlated to invasion and lymph node metastasis of IHCC, and whether slug expression may be a significant prognostic factor after surgical resection.

Terminology

Slug, a member of the snail family of transcription factors, plays a crucial role in regulation of epithelial-mesenchymal transition by suppressing several epithelial markers and adhesion molecules, including E-cadherin.

Peer review

The results of this study support the use of novel biomarkers in diagnosis and treatment of IHCC. The histological identification of slug expression was carefully done and the conclusion is evidence-based.

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BRIEF ARTICLE

High-dose *vs* low-dose proton pump inhibitors for upper gastrointestinal bleeding: A meta-analysis

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Abstract

AIM: To evaluate the efficacy of high-dose proton pump inhibitors (PPIs) *vs* low-dose PPIs for patients with upper gastrointestinal bleeding.

METHODS: PubMed, Embase, the Cochrane Library, and Web of Science were searched to identify relevant randomized controlled trials (RCTs). Eligible trials were RCTs that compared high-dose PPI with low-dose PPI following endoscopic hemostasis. The primary endpoint was rebleeding; secondary endpoints were patient numbers that needed surgery, and mortality. The meta-analysis was performed with a fixed effects model or random effects model.

RESULTS: Nine eligible RCTs including 1342 patients were retrieved. The results showed that high-dose intravenous PPI was not superior to low-dose intravenous PPI in reducing rebleeding [odds ratio (OR) = 1.091, 95% confidential interval (CI): 0.777-1.532], need for surgery (OR = 1.522, 95% CI: 0.643-3.605) and mortality (OR = 1.022, 95% CI: 0.476-2.196). Subgroup analysis according to different region revealed no difference in rebleeding rate between Asian patients (OR = 0.831, 95% CI, 0.467-1.480) and European patients (OR = 1.263, 95% CI: 0.827-1.929).

CONCLUSION: Low-dose intravenous PPI can achieve the same efficacy as high-dose PPI following endoscopic hemostasis.

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Key words: Meta-analysis; High-dose; Low-dose; Proton pump inhibitors; Gastrointestinal bleeding

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INTRODUCTION

Acute upper gastrointestinal bleeding is a prevalent and clinically significant condition[1]. The core management of these patients consists of resuscitation and endoscopic therapy, but rebleeding still occurs after initial control of the bleeding[2]. Currently, the combination of endoscopic

hemostasis and administration of proton pump inhibitors (PPIs) is the standard management, and two authoritative consensus guidelines have recommended the use of a high-dose PPI regimen (80 mg stat followed by an infusion of 8 mg/h for 72 h) following endoscopic hemostasis for high-risk peptic ulcer bleeding lesions[3,4]. The possible biological benefit of this high-dose regimen is to promote clot stability by sustaining the intragastric pH above 6[5,6]. However, when high-dose PPI is compared with a low-dose PPI regimen after primary endocopic hemostasis is achieved, some clinical trials[7-10] reported no differences in rebleeding rate and the need for surgery between the high- and low-dose regimens.

This meta-analysis aims to analyze trials of low-dose *vs* high-dose PPI after endoscopic hemostasis, and to determine whether low-dose PPI administration can produce the same effect as high-dose after endoscopic hemostasis. This study was performed according to the QUOROM (Quality of Reporting Of Meta-analysis) statement, and the QUOROM statement checklists were all answered as "Yes"[11].

MATERIALS AND METHODS

Literature search

A comprehensive literature search was performed to identify all randomized controlled trials (RCTs). Trials were identified by systematically searching the electronic databases PubMed, EMBASE, the Cochrane Library, and Web of Science in any language. The search was conducted using the following key words: omeprazole, lansoprazole, esomeprazole, pantoprazole, dexlansoprazole, rabeprazole, PPI, and bleeding. The last search was done in September 2009. In addition, an extensive manual search was also performed by using references from each retrieved study or review article.

Study selection

Trials were included using the following criteria: (1) RCT; (2) comparing high-dose with low-dose PPI following endoscopic hemostasis for acute upper non-variceal gastrointestinal bleeding; and (3) availability of adequate data (persistent or recurrent bleeding, need for surgery, or mortality). Subsequently, trials were excluded if the PPI treatment was initiated prior to endoscopic hemostasis. The study selection was done by two reviewers independently, and any difference of opinion was settled by discussion. Publications identified as duplicates were excluded, and when one study had substantial overlap in terms of author, institution or study period with another study, the one which was more recent and of better quality was included.

Regarding the definitions of high and low-dose PPIs, high-dose was not limited to the regimen of 80 mg iv bolus followed by an infusion of 8 mg/h for 72 h. In our study, dosage of PPI was considered high-dose if at least twice the low-dose of any of the PPIs was used during the 72 h following endoscopic hemostasis.

Qualitative assessment

The quality of the studies was assessed and graded by two reviewers independently according to criteria described in The Cochrane Handbook 5.0.1[12]. The criteria included: (1) sequence generation; (2) allocation concealment; (3) blinding of participants, personnel and outcome assessors; (4) incomplete outcome data; and (5) selective outcome reporting. Each criteria was categorized as "yes", "no", or "unclear", and the summary assessments of the risk of bias for each important outcome within and across studies was categorized as "low risk of bias", "unclear risk of bias" and "high risk of bias"[12].

Data extraction

Two reviewers independently extracted data by using a standardized form. If there was inconsistency, the original papers were retrieved and jointly investigated to resolve the disagreement. Data were extracted regarding the details of all therapeutic interventions, including the specific kind of PPI, the dose and administration method (oral, intermittent bolus, or continuous infusion). We also extracted the first author, publication year, patients age, sex ratio, region, numbers assigned to each group, numbers with comorbid conditions, the site of bleeding ulcer (duodenal, gastric or esophageal), signs of recent hemorrhage (spurting, oozing, non-bleeding vessel, and adherent clot). Our primary endpoint was persistent or recurrent ulcer bleeding, and secondary endpoints included patient numbers that needed surgery and mortality.

Statistical analysis

The pooled odds ratio (OR) and 95% confidence intervals (CI) were calculated from the original study data by using the Mantel-Haenszel method (fixed effects model) if no heterogeneity was detected throughout the studies[13]; in other words, if there was no variability among the studies in the meta-analysis because all the studies could be assumed to come from the same population. Statistical heterogeneity was assessed using the forest plot and inconsistency statistic (I^2). If any heterogeneity existed ($P < 0.1$, OR $I^2 > 50\%$), then use of the fixed effects model might have been invalid. Any heterogeneity was explored by subgroup analysis and by a sensitivity analysis done by excluding the studies which potentially biased the results. If the investigations of the cause of the heterogeneity showed that this was more appropriate, then the Der Simonian and Laird method (random effects model) was applied. A random effects model assumes that each study has a different underlying effect and this leads to wider confidence intervals than with the fixed effects model[13]. Egger's test was applied to determine the symmetry of the funnel plot, to identify any publication bias, $P < 0.05$ indicated bias, and $P > 0.05$ indicated no publication bias[14].

All data analyses were performed using the statistical software Stata 9.2 (Stata Co., College Station, Texas, USA).

Table 1 Summary assessments of the risk of bias with studies

Author	Yr	Adequate sequence generation?	Allocation concealment?	Blinding?	Incomplete outcome data addressed?	Free of other bias?	Risk of bias
Andriulli *et al*[16]	2008	Yes	Yes	Yes	Yes	Yes	Low
Yüksel *et al*[17]	2008	Yes	Yes	Unclear	Yes	Yes	Low
Hung *et al*[2]	2007	Yes	Unclear	Unclear	No	No	High
Lin *et al*[19]	2006	Unclear	Yes	Unclear	Yes	Unclear	Unclear
Cheng *et al*[21]	2005	Unclear	Yes	Yes	Yes	Unclear	Low
Dokas *et al*[9]	2004	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear
Chilovi *et al*[24]	2003	Unclear	Unclear	Unclear	Unclear	Unclear	High
Udd *et al*[8]	2001	Yes	Yes	Yes	Yes	Unclear	Low
Schonekas *et al*[7]	1999	Unclear	Unclear	Unclear	Unclear	Unclear	High

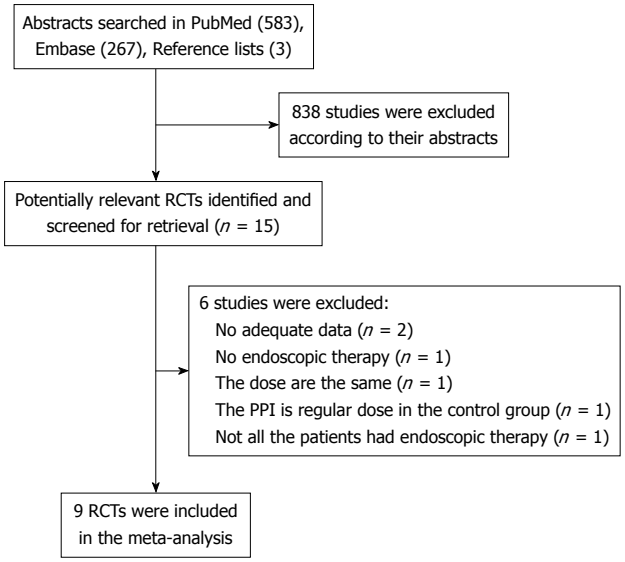


Figure 1 Flow chart of the literature search for this meta-analysis.

RESULTS

Searches of the PubMed and Embase databases identified 583 and 267 abstracts, respectively. After screening the titles and the abstracts, 12 studies from PubMed and Embase and their full texts were retrieved for further review[2,8,10,15-23], and 3 studies were found from reference lists[7,9,24]. Thus, in total, 15 studies were retrieved for further review. Two studies were excluded because no adequate data were available[18,22], one study was excluded because not all the patients received endoscopic therapy[20], one study was excluded because the total dose of PPI was the same across the different groups[23], one study was excluded because the PPI in the intravenous group was regular dose[15], one study was excluded because not all patients had endoscopic intervention[10]. Thus, 9 studies and 3 abstracts[7,9,24] were included in this meta-analysis. The flow chart of the literature search of this meta-analysis is shown in Figure 1.

The qualities of the included studies are shown in Table 1. Thus, 4 studies are at low risk of bias[16,17,21,22], 2 studies are at unclear risk of bias[9,19], and the other 3 studies are at high risk of bias[2,7,24], including two which could not be accessed as full texts. Thus, 4 studies' allocation sequence were adequately generated[2,8,16,17], 5 studies' allocation were adequately concealed[8,16,17,19,21], 3 studies' blinding of participants, personnel and outcome assessors were adequately carried out[8,16,21], 5 studies' incomplete outcome data were adequately addressed[8,16,17,19,21], 2 studies were free of other bias[16,17].

Patients were of European origin in 6 studies[7-9,16,17,24] and Asian in 3 studies[2,19,21]. Baseline characteristics and PPI regimens of the included studies are shown in Tables 2 and 3, respectively. Thus, 9 studies including 1342 patients were analyzed in this meta-analysis, and the mean number of patients per study was 149, ranging from 24 to 474. The mean age of patients was ⩾ 60 years in 6 studies, in the other three studies the age of the patients could not be ascertained. The number of patients who had gastric ulcers was 435, while that of duodenal ulcers was 600, included in the 6 studies from which adequate data could be extracted[2,8,16,17,19,21]. The PPI used in the included studies was omeprazole or pantoprazole. In fact, 4 studies used omeprazole[8,9,19,21], 3 studies used pantoprazole[2,7,17], while the other 2 studies used both drugs[16,24].

Rebleeding

Nine studies including 1342 patients evaluated the rate of rebleeding as an outcome measure; 666 patients in the high-dose group and 676 patients in the low-dose group. The summary OR for rebleeding was 1.091 (95% CI: 0.777-1.532, $P = 0.617$) suggesting the result was not statistically significant when comparing high-dose with low-dose PPI following endoscopic hemostasis in reducing the risk of rebleeding (Figure 2A). The χ^2 test for heterogeneity for the high-dose effect in rebleeding was not significant ($I^2 = 0.0\%$, $P = 0.498$). The subgroup analysis according to different region did not indicate any benefit in either European or Asian population with high-dose PPI regimen compared with low-dose regimen following endoscopic hemostasis; for Asian patients the summary OR was 0.831 (95% CI: 0.467-1.480, $P = 0.529$), while for European patients the summary OR was 1.263 (95% CI: 0.827-1.929, $P = 0.279$). No heterogeneity was detected in those two subgroup analyses, with *P*-values of 0.143 and 0.812, respectively. The summary OR for rebleeding from 4 studies using omeprazole[8,9,19,21] (OR = 0.926, 95% CI: 0.550-1.557) and for the remaining 5 studies using pantoprazole/omeprazole[2,7,16,17,24]

Table 2 Baseline characteristics of the included studies

Author	Yr	Region	Arm	No.	Age (yr)	Male/ female	NSAID	Aspirin	Antico-agulant	*H. pylori* positive	Comorbid disease	Gastric ulcer	Duodenal ulcer	Other ulcer[3]	Study population
Andriulli *et al*[16]	2008	European	High	238	66.3 ± 15.6	151/87	75	72	NA	NA	19	88	150	0	High-risk group[1]
			Low	236	66.8 ± 16.7	156/80	74	82	NA	NA	30	93	143	0	
Yüksel *et al*[17]	2008	European	High	48	60.4 ± 14.0	38/10	19	22	NA	NA	21	16	32	0	High-risk group[1]
			Low	49	56.3 ± 17.6	36/13	20	25	NA	NA	16	16	33	1	
Hung *et al*[2]	2007	Asia	High	54	63.7	32/22	6	9	NA	30	NA	19	35	0	High-risk group[1]
			Low	49	57.8	35/14	6	5	NA	33	NA	14	35	0	
Lin *et al*[19]	2006	Asia	High	67	67	58/9	NA	NA	NA	48	53	26	35	6	High-risk group[1]
			Low	66	71	57/9	NA	NA	NA	51	50	29	33	4	
Cheng *et al*[21]	2005	Asia	High	52	62.5 ± 12.5	36/16	13	1	2	27	52	28	19	5	Comorbid illnesses
			Low	53	65.8 ± 13.8	31/22	12	3	1	21	53	29	20	4	
Dokas *et al*[9]	2004	European	High	10	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	High-risk group[1]
			Low	14	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	
Chilovi *et al*[24]	2003	European	High	46	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			Low	45	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	
Udd *et al*[8]	2001	European	High	69	63.4 ± 15.7	41/28	37	7	10	46	55	39	30	0	Unselected[2]
			Low	73	66.0 ± 13.3	44/29	41	16	4	45	54	38	35	0	
Schonekas *et al*[7]	1999	European	High	82	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			Low	86	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	

[1]Included peptic ulcer patiens with spurting artery, ooze, non-bleeding visible vessel or adherent clot; [2]Included peptic peptic ulcer patiens with spurting artery, ooze, non-bleeding visible vessel, adherent clot or black base; [3]Include both gastral and duodenal ulcer, esophagal ulcer. *H. pylori*: *Helicobacter pylori*; NSAID: Non-steroidal anti-inflammatory drug; NA: Not available.

Table 3 Proton pump inhibitor regimen of the included studies

Author	Arm	Endoscopic therapy	Schedule of the PPIs
Andriulli *et al*[16]	High	Epinephrine injection or thermal or mechanical therapy	OME or PAN 80 mg iv bolus, 8 mg/h infusion × 3 d
	Low		OME or PAN 40 mg iv bolus 24 h × 3 d
Yüksel *et al*[17]	High	Epinephrine injection and thermal therapy	PAN 80 mg iv bolus, 8 mg/h infusion × 3 d
	Low		PAN 40 mg iv bolus Q12 h × 3 d
Hung *et al*[2]	High	Epinephrine injection or thermal therapy	PAN 80 mg iv bolus, 8 mg/h infusion × 3 d
	Low		PAN 80 mg iv bolus, 40 mg iv bolus Q12 h × 3 d
Lin *et al*[19]	High	Epinephrine injection	OME 40 mg infusion Q6 h × 3 d
	Low		OME 40 mg infusion Q12 h × 3 d
Cheng *et al*[21]	High	Epinephrine injection or thermal therapy	OME 80 mg iv bolus, 200 mg infusion 24 h × 3 d
	Low		OME 80 mg iv bolus, 80 mg infusion 24 h × 3 d
Dokas *et al*[9]	High	Epinephrine injection or mechanical therapy	OME 80 mg iv bolus, 160 mg infusion Q24 h × 3 d
	Low		OME 40 mg iv bolus Q12 h × 3 d
Chilovi *et al*[24]	High	NA	PAN 80 mg iv bolus, 40 mg iv bolus Q8 h
	Low		OME 80 mg iv bolus, 40 mg
Udd *et al*[8]	High	Epinephrine injection or thermal or mechanical therapy	OME 80 mg iv bolus, 8 mg/h infusion × 3 d
	Low		OME 20 mg iv bolus 24 h × 3 d
Schonekas *et al*[7]	High	NA	PAN 40 mg iv bolus 8 mg/h infusion × 3 d
	Low		PAN 40 mg iv bolus Q24 h × 3 d

PPIs: Proton pump inhibitors; OME: Omeprazole; PAN: Pantoprazole.

(OR = 1.234, 95% CI: 0.786-1.936) did not differ in rebleeding rate. The summary OR for rebleeding from 5 studies[2,9,16,17,19] with endoscopic high-risk stigmata for rebleeding revealed no reduction in the high-dose PPI group (OR = 0.991, 95% CI: 0.628-1.565, P = 0.970). The summary OR for rebleeding from 4 studies[16,17,21,22] with low risk of bias revealed no reduction in the high-dose PPI group (OR = 1.479, 95% CI: 0.963-2.269, P = 0.999). When excluding the 3 abstracts, the summary OR for rebleeding from the remaining 6 studies revealed no reduction in the high-dose PPI group (OR = 1.130, 95% CI: 0.827-1.544, P = 0.335). In addition, the time-frame of rebleeding rate should be taken into consideration, because one study reported early rebleeding (within first 72 h)[17], two studies reported in-hospital occurrence of rebleeding[8,25], one study reported rebleeding before discharge and within 14 d[19], and 2 studies reported rebleeding within 30 d[21,26]; the time-frame of rebleeding was not reported in the remaining 3 studies[7,9,24].

Surgery

Seven studies[2,8,9,16,17,19,24] including 1225 patients evaluated the rate of surgery as an outcome measure; 532 patients in the high-dose group and 537 in the low-dose group. Thus, the need for surgery was reported in 2.3% of the patients treated with high-dose PPI, and in 1.5%

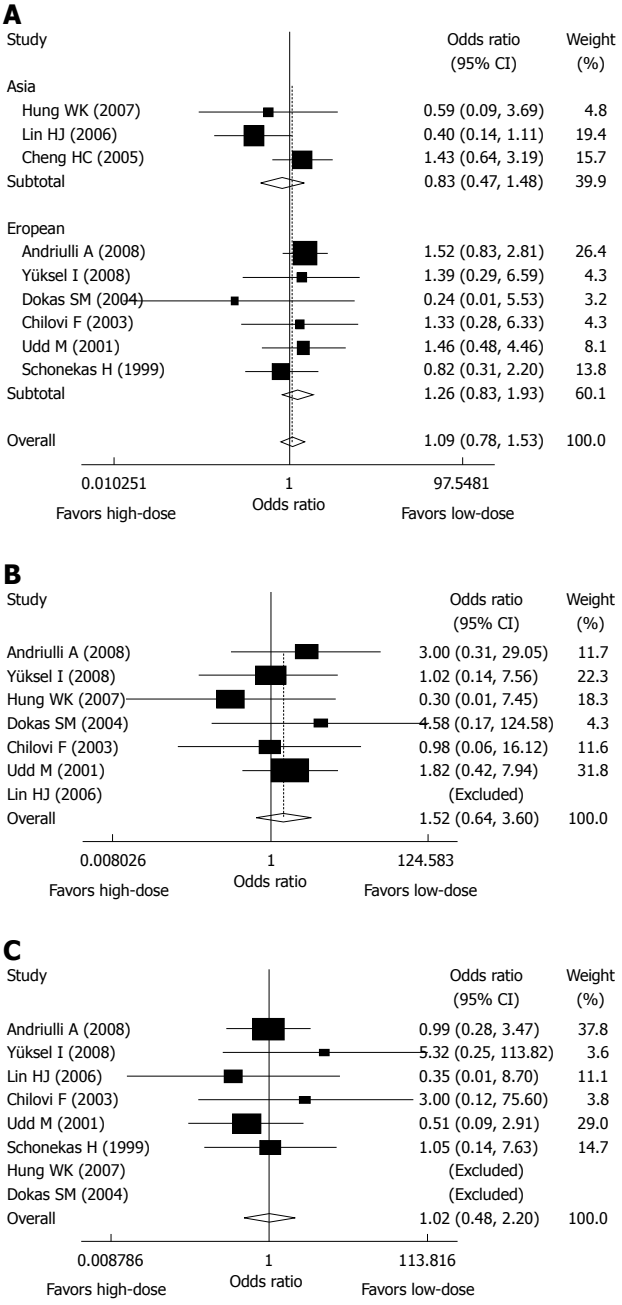


Figure 2 Meta-analysis results of randomized clinical trials comparing high-dose proton pump inhibitor (PPI) *vs* low-dose PPI in rebleeding (A), surgery (B) and mortality (C).

of the patients treated with low-dose PPI. The summary OR for surgery was 1.522 (95% CI: 0.643-3.605, P = 0.340), indicating the result was not statistically significant when comparing high-dose with low-dose PPI following endoscopic hemostasis in reducing the need for surgery (Figure 2B). The χ^2 test for heterogeneity for the high-dose effect in reducing the need for surgery was not significant (I^2 = 0.0%, P = 0.840). The summary OR for surgery from 5 studies[2,9,16,17,19] with endoscopic high-risk stigmata for rebleeding revealed no reduction in the high-dose PPI group (OR = 1.465, 95% CI: 0.461-4.655, P = 0.517). The summary OR for surgery from 4 studies[16,17,21,22] with low risk of bias revealed no reduction in the high-dose PPI group (OR = 1.760, 95% CI: 0.626-4.946, P = 0.780). When excluding the 2 abstracts, the summary OR for surgery from the remaining 5 studies revealed no reduction in the high-dose PPI group (OR = 1.422, 95% CI: 0.569-3.556, P = 0.676).

Mortality

Eight studies[2,7-9,16,17,19,24] including 1237 patients evaluated the rate of mortality as an outcome measure; 614 patients in the high-dose group and 623 patients in the low-dose group. Thus, mortality was reported in 2.0% of the patients treated with high-dose PPI, and in 1.9% of the patients treated with low-dose PPI. The summary OR for mortality was 1.022 (95% CI: 0.476-2.196, P = 0.955), indicating the result was not statistically significant comparing high-dose with low-dose PPI following endoscopic hemostasis in reducing overall mortality (Figure 2C). The χ^2 test for heterogeneity for the high-dose effect of affecting overall mortality was not significant (I^2 = 0.0%, P = 0.765). Six studies[2,8,9,16,17,19] reported causes of death, and rebleeding caused 8 deaths (4 in the high-dose PPI group and 4 in the low-dose group); the summary OR for rebleeding-related deaths was 1.008 (95% CI: 0.250-4.067, P = 0.991), suggesting no benefit with high-dose PPI therapy. No studies stated the time-frame of mortality rate clearly, so they were assumed to be in-hospital mortality rates.

Publication bias

The P-values from Egger's test in rebleeding, the need for surgery, and mortality were of 0.183, 0.674, and 0.488, respectively. Thus, no publication bias exists in this meta-analysis.

DISCUSSION

Rebleeding has remained the most important adverse prognostic factor contributing to morbidity and mortality after upper gastrointestinal hemorrhage[25]. Previous consensus groups have recommended that an intravenous bolus followed by continuous-infusion PPI is effective in decreasing rebleeding in patients who have undergone successful endoscopic therapy[4]. However, this recommendation of a high-dose of PPI is in conflict with some previous studies[7,16], which reported no difference in the magnitude of risk reduction between high-dose and low-dose regimens. Therefore, this meta-analysis set out to evaluate the efficacy of a high-dose PPI regimen *vs* a low-dose PPI regimen in reducing rebleeding, the need for surgery and the mortality rate following endoscopic hemostasis for upper gastrointestinal bleeding.

The findings of this meta-analysis suggest that a high-dose PPI regimen for patients who have undergone successful endoscopic therapy is not superior to a low-dose PPI regimen for the parameters of rebleeding (OR = 1.091, 95% CI: 0.777-1.532, P = 0.617), the need for surgery (OR = 1.522, 95% CI: 0.643-3.605, P = 0.340),

and mortality (OR = 1.022, 95% CI: 0.476-2.196, P = 0.955) and rebleeding-related deaths (OR = 1.008, 95% CI: 0.250-4.067). Also, no heterogeneity exists, suggesting that the studies for these endpoints were homogenous.

It has been hypothesized that the efficacy of a high-dose PPI regimen may vary depending on physiological measures, pharmacodynamic profiles (metabolism of proton-pump inhibitors through the cytochrome P4502C19 genetic polymorphism), and prevalence rates of *Helicobacter pylori* infection in different ethnicities[26], so subgroup analysis was carried out to determine whether low-dose PPI can have the same efficacy in patients from different regions. This subgroup analysis did not find any difference in rebleeding rate between Asian and European patients using a low-dose PPI regimen. However, the need for surgery and mortality cannot be assessed in this subgroup analysis due to limited studies. Thus, a low-dose PPI regimen can achieve the same efficacy in reducing rebleeding as a high-dose regimen in both Asian (OR = 0.831, 95% CI: 0.467-1.480, P = 0.529) and European patients (OR = 1.263, 95% CI: 0.827-1.929, P = 0.279). The endoscopic therapy technique was epinephrine injection, plus if indicated thermal or mechanical therapy, and this technique was applied in 5 studies[2,8,9,16,21]. This endoscopic therapy technique is practical and widely used in most hospitals, so the endoscopic therapy technique was homogenous across the included studies. In this present meta-analysis, four studies evaluated omeprazole, while the other 5 studies evaluated pantoprazole or both drugs together. Subgroup analysis according to different PPI showed similar results to those found in all 9 studies, so this effect of PPI seems to be a class effect.

This meta-analysis showed that the high-dose PPI regimen did not affect overall mortality (OR = 1.022, 95% CI: 0.476-2.196, P = 0.955) when compared with the low-dose PPI regimen. A total of 8 deaths were rebleeding-related (50% in the high-dose group and 50% in the low-dose group), and 11 deaths were non-ulcer deaths (45.5% in the high-dose group and 54.5% in the low-dose group). The data in this present meta-analysis indicated that the high-dose group had a similar mortality rate as the low-dose group independent of the outcome of comorbid diseases. However, this mortality result is not reliable, because it has been estimated that in order to detect clinically important effects on mortality, at least 1000 high-risk patients should be included in each of the treatment and control groups[27]; thus the number of high-risk patients included in this meta-analysis is not enough. Nonetheless, since high-dose regimens can produce similar reductions in rebleeding and need for surgery as low-dose regimens, maybe the effect of a high-dose PPI regimen on mortality is the same as a low-dose PPI regimen.

Summary improvements in transfusion requirements and duration of hospital stay have not been analyzed in this meta-analysis, because these improvements are of lesser clinical magnitude and are based on weaker source study methodology[28].

A previous meta-analysis revealed that PPI therapy is only efficacious for patients who had endoscopic high-risk stigmata for rebleeding[29]. In addition, subgroup analysis has shown that in this particular patient group, low-dose PPI is as effective as high-dose PPI. Future studies should stratify the management of patients according to low-, intermediate-, and high-risk endoscopic lesions with relevant strategies that include low-dose PPI regimens.

Cost-effectiveness analyses using decision modeling and direct costing from studies have demonstrated that the use of high-dose PPI following endoscopic hemostasis saves money for health care systems in most clinical scenarios[30,31]. Thus, if low-dose PPI is as effective as high-dose, it is assumed that this can result in savings in health care costs. The most recent randomized trials comparing the use of intravenous regular-dose omeprazole with oral rabeprazole revealed that these drugs are equally effective in preventing rebleeding in patients with high-risk bleeding peptic ulcers after successful endoscopic injection with epinephrine[15]. If intravenous PPIs could be replaced by oral PPIs, this could result in significant savings in healthcare resources[32]. This trial was performed in an Asian population, however, so additional data from randomized clinical trials comparing the use of intravenous PPIs with that of oral PPIs are required in western patient populations.

In this meta-analysis, the modality of endoscopic intervention and the type of PPI used have varied across studies. Regarding the claim that monotherapy with epinephrine is inferior to dual endoscopic therapies[33], published data are not consistent. Pharmacologic data indicate a class effect of PPI therapy as inhibitors of gastric secretion, and one study did find similar rebleeding rates in a comparative trial of omeprazole *vs* pantoprazole[24]. Therefore, the differences in practice patterns across different studies might constitute a particular strength of this meta-analysis, because it better approximates the real-life practice due to availability of different PPIs and endoscopic interventions in different hospitals. Thus, the differences in the modality of endoscopic intervention and the type of PPI would not have affected the overall validity of our findings.

There are some limitations in this meta-analysis: firstly, a total of nine studies were included, in which only four studies were at low risk of bias, and three studies could not be accessed as full texts, so the included studies are not quite adequate; secondly, although subgroup analysis revealed that the summary OR for rebleeding remained statistically stable (similar to summary OR obtained from 9 studies), it does not prove the stability of our results; thirdly, the time-frame of rebleeding rate varied between different studies, so the results for rebleeding should be interpreted with caution, and further subgroup analyses were not performed in this meta-analysis; fourthly, studies included in this meta-analysis were carried out with varying doses of PPI, different populations with different risks of rebleeding and varying proportions of associated comorbid diseases. It is inevitable

in clinical practice to encounter different patients, so this should not affect the overall validity of the findings.

In conclusion, a low-dose PPI regimen is as efficacious as a high-dose PPI regimen following endoscopic hemostasis in upper gastrointestinal bleeding patients, and this beneficial effect seems to be similar in both Asian and European patients. Future comparative trials are warranted to compare oral PPI with intravenous PPI following endoscopic hemostasis in western patient populations.

COMMENTS

Background

The combination of endoscopic hemostasis and the administration of proton pump inhibitors (PPIs) is the current standard management for upper gastrointestinal hemorrhage. A low-dose of PPIs may achieve the same efficacy as high-dose PPIs following endoscopic hemostasis.

Research frontiers

This meta-analysis investigated the rebleeding rate, the need for surgery and mortality outcomes in randomized controlled trials (up to 9 studies).

Innovations and breakthroughs

Compared with high-dose PPIs following endoscopic hemostasis, low-dose PPIs can achieve the same effect in reducing rebleeding, the need for surgery and mortality. This beneficial effect seems to be consistent in both Asian and European patients.

Applications

The study can be applied as guidance for using low-dose PPI regimens following endoscopic hemostasis for gastrointestinal bleeding.

Peer review

This is a good quality work on a subject of everlasting interest.

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CASE REPORT

Intestinal amyloidosis: Two cases with different patterns of clinical and imaging presentation

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Abstract

The involvement of the small bowel in systemic forms of amyloidosis may be diffuse or very rarely focal. Some cases of focal amyloidomas of the duodenum and jejunum without extraintestinal manifestations have been reported. The focal amyloidomas consisted of extensive amyloid infiltration of the entire intestinal wall thickness. Radiological barium studies, ultrasound and computed tomography (CT) patterns of diffuse small bowel amyloidosis have been described: the signs are non-specific and may include small-bowel dilatation, symmetric bowel wall thickening, mesenteric infiltration, and mesenteric adenopathy. No data are available about the positron emission tomography (PET)/CT and magnetic resonance imaging (MRI) patterns of intestinal amyloidosis. We report two cases of small bowel amyloidosis: the former characterized by focal deposition of amyloid proteins exclusively within blood vessel walls of the terminal ileum, the latter characterized by diffuse intestinal involvement observed on MRI and PET/CT studies.

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Key words: Amyloidosis; Small bowel; Positron emission tomography/computed tomography; Magnetic resonance imaging; Computed tomography

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INTRODUCTION

Systemic amyloidosis is a rare disease characterized by the extracellular deposition of amyloid protein in one or more organs[1]. Involvement of the small bowel is frequent in systemic amyloidosis[2], and protein deposition

is mainly seen in the mucosa, the submucosal connective tissues, the muscularis propria and within blood vessel walls. The deposition of amyloid into the blood vessel walls causes bowel ischemia and muscle atrophy leading, eventually, to mucosal erosion or ulceration[3].

The involvement of small bowel may be diffuse or very rarely localized, presenting as focal amyloidoma[4-6]. Focal amyloidomas of the small bowel without extraintestinal manifestations are rare and typically present as multiple polypoid lesions or large amyloid tumors of the duodenum and jejunum[5-7].

To the best of our knowledge, a case of focal deposition of amyloid proteins exclusively within blood vessel walls of the terminal ileum has never been described in the literature.

Radiological barium studies, ultrasound (US) and computed tomography (CT) pattern of diffuse small bowel amyloidosis have been described: the signs are non-specific and may include small-bowel dilatation, symmetric bowel wall thickening, mesenteric infiltration, and mesenteric adenopathy[4,8,9].

On the contrary, the positron emission tomography (PET)/CT and magnetic resonance imaging (MRI) patterns of intestinal amyloidosis have never been described in the literature.

We report two cases of small bowel amyloidosis: the former characterized by focal deposition of amyloid proteins exclusively within blood vessel walls of the terminal ileum, the latter characterized by diffuse intestinal involvement observed on PET/CT and MRI studies.

CASE REPORT

Case 1

A 72-year-old woman with a medical history of hypertension and of multiple episodes of acute onset of abdominal pain accompanied by intestinal sub-occlusion presented in our hospital.

Two months before our observation, the patient had undergone conventional colonoscopy with retrograde ileoscopy, which disclosed mucosal ulcerations of the terminal ileum and of the ileo-cecal valve. The biopsy specimen showed active inflammatory infiltration without evidence of granulomas.

On admission the patient had acute abdominal pain and intestinal sub-occlusion. She was afebrile with stable vital signs. Findings on physical examination were significant for distended abdomen with discrete tenderness in the right iliac fossa.

Laboratory studies including complete blood count, serum electrolytes, liver function tests, coagulation and tumour markers were as follows (with normal values): hematocrit, 34% (37%-54%); haemoglobin, 11.5 g/dL (12-16); white blood cell count, 16 300 cells/mm^3 (4800-10 800); alkaline phosphatase, 117 U/L (35-104); γ-glutamyl transpeptidase, 81 U/L (5-36); albumin, 3.5 g/dL (3.6-5.2). The remaining values were unremarkable.

An US examination of the abdomen showed thickened (20 mm) hypoechoic walls of the terminal ileum with loss of parietal stratification and mild pseudoaneurysmal dilatation of the lumen. Moreover multiple ileo-cecal lymph nodes with a diameter ranging between 10 and 20 mm were disclosed.

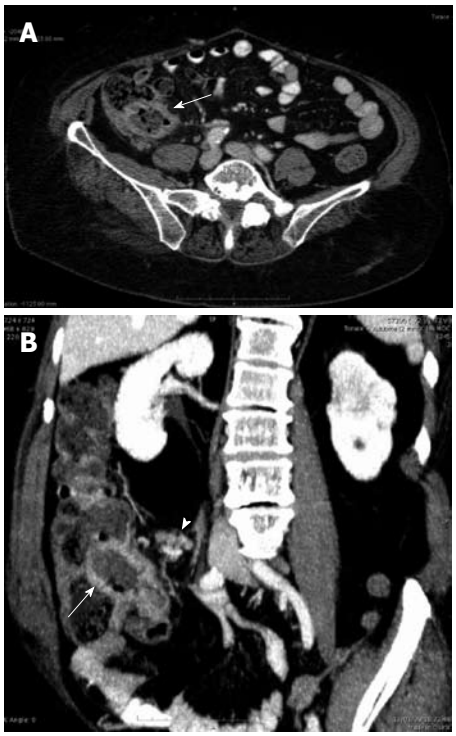


Figure 1 The axial computed tomography (CT) image (A) and the multiplanar reformatted (MPR) coronal oblique image (B) showed thickened walls of the terminal ileum with mild pseudoaneurysmal dilatation of the lumen (arrows) and multiple ileo-cecal lymph nodes (arrow head).

To better evaluate the US intestinal findings, a 64 multidetector CT scan of the abdomen and pelvis was performed 75 s after the intravenous bolus administration of 150 cc iodinated non ionic contrast agent. The CT images confirmed the findings of US (Figure 1A and B). The mesenteric vessels and the collateral branches were patent.

The US and CT findings suggested the following diagnostic hypothesis: an intestinal lymphoma, considering the pseudoaneurysmal dilatation of the intestinal lumen and the dimension of ileo-cecal lymph nodes or less probably a terminal ileitis. Features of intestinal ischemia were not present.

The patient underwent laparotomy with ileo-caecal resection. The biopsy specimen was consistent with a deposition of amyloid within blood vessels of the terminal ileum walls after staining with Congo red (Figure 2).

The patient was discharged from the hospital in a stable condition 5 d after surgery. Further workup showed (1) a monoclonal spike in the serum immunoelectrophoreses; (2) the immunoglobulin κ light-chain urine concentration was 71 mg/L (normal, < 10 mg/L) and the immunoglobulin λ light-chain urine concentration was 26 mg/L (normal, < 5 mg/L); and (3) 8% plasma cells in the bone marrow aspirate. There was no clinical, laboratory, or radiographic evidence of extraintestinal involvement of

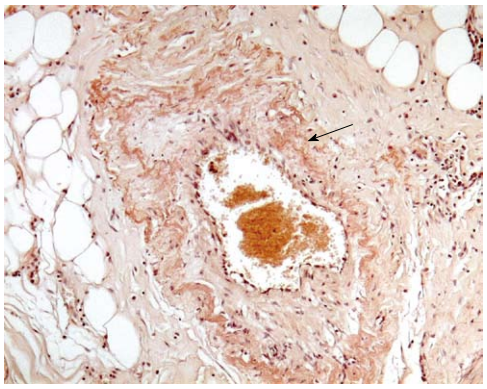


Figure 2 Intestinal amyloid ("congophilic") angiopathy. Amyloid in the intramural vessels of terminal ileum walls (arrow) takes the Congo red stains (× 270).

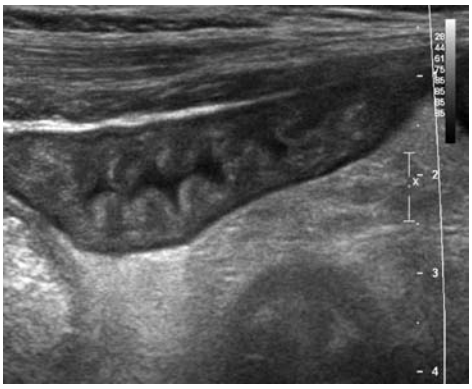


Figure 3 Ultrasound images. Thickened and stratified bowel walls with hypertrophy of the mucosal and sub-mucosal strata and increased number of folds due to amyloid deposition.

amyloidosis. These examinations suggested the diagnosis of primary systemic amyloidosis (AL) with focal intestinal manifestation.

Case 2

In March 2009, a 78-year-old man with a medical history of multiple myeloma and diabetes mellitus presented with diffuse abdominal pain, nausea, lack of appetite and diarrhoea. The patient had a recent episode of rectorrhage.

On admission the patient was afebrile with stable vital signs. Findings on physical examination were significant for moderately distended abdomen with mild diffuse tenderness.

On admission, laboratory studies including complete blood count, serum electrolytes, liver function tests, coagulation and tumour markers were as follows (with normal values): hematocrit, 27% (37%-54%); red blood cell count, 3 780 000 cells/mm^3 (4 200 000-5 600 000); haemoglobin, 8.4 g/dL (12-17.5); white blood cell count, 2333 cells/mm^3 (4800-10 800); glucose, 177 mg/dL (60-110); total protein, 5.6 g/dL (6.5-8.2); albumin, 3.3 g/dL (3.6-5.2). The remaining values were unremarkable.

Urinalysis showed 100 mg/dL of proteinuria. Serum immunoelectrophoreses showed a monoclonal spike. In the urine, the immunoglobulin λ light-chain concentration was 929 mg/L (normal, < 5 mg/L) and the immunoglobulin κ light-chain concentration was 37.8 mg/L (normal, < 10 mg/L).

An US examination of the abdomen showed thickened and stratified intestinal walls with hypertrophy of mucosal and sub-mucosal strata and increased number of plicae which were forming a polyp-like shape (Figure 3).

To better evaluate the US intestinal findings, an MRI of the small bowel previous gastro-intestinal preparation with 750 mL of polyethylene glycol solution as an oral contrast agent was performed using a 3.0 T MR device and the following acquisition protocol: axial and coronal T2-weighted half-Fourier single-shot turbo spin-echo sequences and true fast imaging in the steady-state precession sequences, fast low-angle shot T1-weighted sequences with fat suppression, followed by "spoiled" 3D T1-volume interpolated breath-hold examination acquired before and 70 s after intravenous administration of gadopentetate dimeglumine (0.1 mmol/kg).

The MR images showed diffuse intestinal wall symmetrical thickening with increased number and hypertrophy of the folds causing "jejunalization" of the ileum; wall stratification was not observed. After intravenous administration of contrast agent, diffuse enhancement of intestinal walls was observed (Figure 4A-C). The wall thickening associated with the increased number and hypertrophy of the folds suggested a malabsorption disease. The enhanced images did not show any sign indicative of a specific pathology. As a consequence it was not possible to perform a definitive diagnosis exclusively on the basis of MR findings.

Moreover, a PET/CT scan was performed to re-stage the multiple myeloma as requested by the haematologist. After a fast of 6 h prior to examination and previous normalization of the blood glucose levels with an oral hypoglycemic agent, PET/CT examination was carried out 60 min after intravenous administration of 370 MBq of 18FDG. The PET/CT images did not show a focal or diffuse increase in tracer uptake of small bowel walls (Figure 5).

Finally, the US, MRI and PET/CT findings suggested a malabsorption disease. As a result, considering that the patient was affected by myeloma, the diagnosis of primary systemic AL involving diffusely the gastro-intestinal tract was evaluated as the most probable. To test this diagnostic hypothesis we performed a biopsy of the rectal mucosa: the histological examination of the specimen disclosed diffuse infiltration by amyloid after staining with Congo red (Figure 6).

DISCUSSION

Focal amyloidomas of the duodenum and jejunum have been described as extensive amyloid deposits involving the entire wall thickness, with destruction of the mucosa and deep infiltration into the muscularis propria and with luminal narrowing of blood vessels[4-7].

The peculiarity of case report 1 is related to the focal deposition of amyloid proteins exclusively within intramural blood vessel walls of the terminal ileum. In our patient arteriolar amyloid deposition may have caused

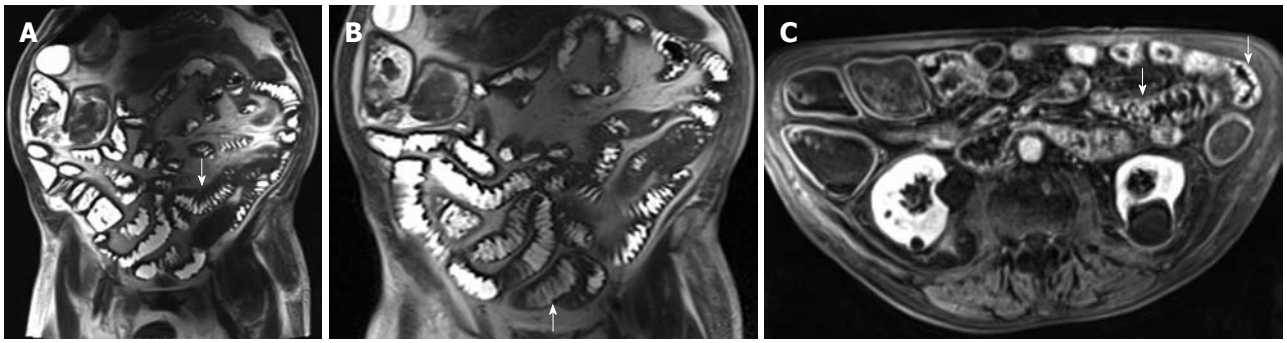


Figure 4 MRI changes of the small bowel. A and B: Coronal T2-weighted half-Fourier single-shot turbo spin-echo magnetic resonance images showed diffuse intestinal wall symmetrical thickening with increased number and hypertrophy of the folds (A, arrow) and "jejunalization" of the ileum (B, arrow); C: 3D T1-fast imaging in the steady-state precession after iv administration of contrast agent showed diffuse enhancement of intestinal walls (arrows).

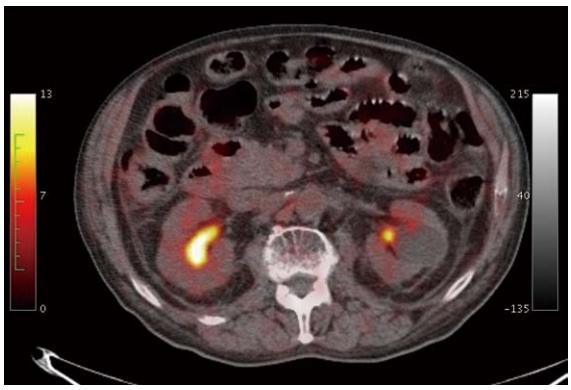


Figure 5 18FDG-positron emission tomography (PET)/CT images did not show focal or diffuse increase of the tracer uptake of small bowel walls.

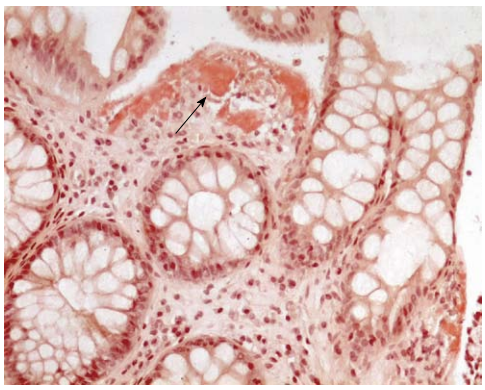


Figure 6 Colonic biopsy. Submucosal amyloid deposition (arrow) (Congo red stains, × 270).

ischemia, resulting pathologically in submucosal edema and mucosal erosion and ulceration and small bowel wall thickening, and clinically in acute onset of abdominal pain with intestinal sub-occlusion.

The thickened walls of the terminal ileum with mild pseudoaneurysmatic dilatation of the lumen associated to multiple ileo-cecal lymph nodes ranging between 10 and 20 mm suggested to us an intestinal lymphoma or less probably an atypical presentation of a terminal ileitis. The hypothesis of intestinal ischemia related to focal amyloid deposition was not evaluated.

Although a specific US or CT pattern has never been reported, the presence of a focal pseudoaneurysmal dilatation of the small bowel lumen associated to wall thickening may represent a radiological sign useful for considering intestinal amyloidosis in the differential diagnosis[4,8,9]. Amyloid may form deposits within the neural myenteric plexus, which determine the hypotonia of the gastro-intestinal wall and promote the pseudoaneurysmal dilatation[9]. On the contrary, the segmental bowel loop pseudoaneurysmal dilatation of intestinal lymphoma is highly suggestive of underlying celiac disease, likely as a reflection of the generalized hypotonia known to exist in the presence of this condition[10].

The diagnosis of localized intestinal amyloidosis is (1) potentially problematic because it is possible that the pathology may occur in the setting of subclinical extraintestinal disease or may represent an early manifestation of systemic amyloidosis; and (2) really crucial because the prognosis for patients with focal amyloidosis is generally good, whereas that for patients with systemic disease is usually poor[1]. Therefore, since the clinical presentation is often not specific and an abdominal CT scan may be required, it is important that radiologists consider the possible diagnosis of amyloidosis in the presence of focal pseudoaneurysmal dilatation of the small bowel lumen associated with wall thickening.

Case report 2 is the first describing the MRI findings of diffuse intestinal amyloidosis characterized by (1) wall symmetrical thickening with increased number and hypertrophy of the folds causing "jejunalization" of the ileum; (2) the absence of wall stratification; and (3) diffuse non-specific wall enhancement and the PET/CT findings characterized by the absence of a focal or diffuse increase of tracer uptake in diffuse thickened small bowel walls.

MRI signs, like the US and CT patterns, seem to be non-specific and are common to other malabsorption diseases, however they may suggest the clinician should consider diffuse intestinal amyloidosis in the differential diagnosis. On the other hand, the lack of pathological tracer uptake on PET/CT images is highly indicative of the absence of active inflammatory and/or neoplastic cells in the intestinal walls and represents a really important finding suggesting the storage nature of the disease char-

acterized by the intramural deposition of an amorphous substance such as amyloid. Considering that a PET/CT scan may be performed in the staging or restaging protocol of patients with multiple myeloma, the radiologist and the nuclear medicine physician should suggest the coexistence of primary systemic AL when they observe absence of pathological tracer uptake in a diffuse intestinal wall thickening.

In conclusion, since the clinical presentation of focal or diffuse intestinal amyloidosis is frequently non-specific and requires an US and/or CT and/or MRI scan and occasionally a PET/CT scan, it is important that radiologists become confident with imaging findings of intestinal amyloidosis in order to make a correct differential diagnosis allowing a proper diagnostic and therapeutic management.

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CASE REPORT

A case of intrahepatic clear cell cholangiocarcinoma

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Abstract

Intrahepatic clear cell cholangiocarcinoma is very rare - only 8 cases have been reported. A 56-year-old Japanese man with chronic hepatitis B infection was diagnosed with a 2.2 cm hepatocellular carcinoma on imaging, and hepatic segmentectomy was performed. Histopathologically, the tumor cells had copious clear cytoplasm and formed glandular structures or solid nests. These pathological findings suggested the tumor was a clear cell variant of intrahepatic cholangiocarcinoma. Particular stains and radiological images suggested that the cause of the clear cell change had been glycogen, not mucin nor lipid. On immunohistochemical staining, cytokeratin (CK) 7 and CK19 were positive, whereas CK20 was negative. Vimentin was detected on the cell membranes, and CD56 was focally positive. The patient was given adjuvant chemotherapy and is currently free from the tumor 7 mo postoperatively. Careful follow-up with adequate postoperative supplementary chemotherapy is necessary because the characteristics of this type of tumor are unknown.

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Key words: Intrahepatic cholangiocarcinoma; Clear cell carcinoma; Glycogen; Mucin; Lipid; Hepatectomy

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Toriyama E, Nanashima A, Hayashi H, Abe K, Kinoshita N, Yuge S, Nagayasu T, Uetani M, Hayashi T. A case of intrahepatic clear cell cholangiocarcinoma. *World J Gastroenterol* 2010; 16(20): 2571-2576 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i20/2571.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i20.2571

INTRODUCTION

Intrahepatic cholangiocarcinoma (ICC) represents about 15% of primary liver cancers[1]. Several studies in recent years have suggested that chronic hepatitis increases the carcinogenesis of ICC, with particular relevance of hepatitis B virus infection[2,3]. Therefore, it is often difficult to preoperatively distinguish ICC from hepatocellular carcinoma (HCC), despite the high resolution of the latest imaging techniques[3].

In ICC, there is a quite rare variant, clear cell carcinoma[1,4-11]; until now, only 8 cases have been reported[4,7-11] (Table 1). The tumor is characterized by proliferation of the tumor cells with clear cytoplasm similar to renal clear cell carcinoma. The patients are in their 50 s or 60 s and no gender specificity has been found. The patients often have no underlying diseases. The background and risk factors of the ICC clear cell variant are unknown. Unlike

typical ICC, there is no report on the relevance of hepatitis. The prognosis is relatively good. The mechanism for the clear cell change has been speculated to involve glycogen[4,7], mucin[7-9], or lipid[4,7], but it remains unclear.

In this report, a case of intrahepatic clear cell cholangiocarcinoma with background chronic hepatitis B infection is presented. We discuss histopathological and radiological findings on the mechanism of clear cell change, and present a review of the literature.

CASE REPORT

A 56-year-old Japanese man who was found to have hepatic dysfunction on annual examination 3 years previously. He was diagnosed as having chronic hepatitis B (HBe-antigen (Ag)-positive and HBe-antibody (Ab)-negative). He was started on antiviral treatment with lamivudine {(-)-1-[(2R,5S)-2-hydroxymethyl-1,3-oxathiolan-5-yl]}. As the antiviral effect was not adequate, he was changed to a new drug, entecavir hydrate {9-[(1S,3R,4S)-4-hydroxy-3-(hydroxymethyl)-2-metylenecyclopentyl] guanine monohydrate cytosine}, to prevent tolerance emerging. As a result, HBV-DNA became negative, but the amplification signal of the virus remained detectable.

Follow-up abdominal ultrasonography 1 mo prior to surgery revealed a well-circumscribed, 2 cm mass with a halo at segment 7 (S7) of the liver. The tumor was diagnosed as HCC based on the findings of computed tomography (CT) and magnetic resonance imaging (MRI) of the abdomen. He was admitted to our hospital for surgery. At the time of admission, laboratory examination (Table 2) showed that α-fetoprotein (AFP) and protein induced by vitamin K antagonist or agonist (PIVKA)-Ⅱ were in the normal range, and liver function tests were mildly abnormal (Child-Pugh Class A). Both HBs-Ag and HBs-Ab were high.

CT showed a well circumscribed mass in the postero-superior S7 of the right lobe, measuring approximately 2 cm in diameter, with no calcification or fatty component (Figure 1A). After intravenous injection of iopamidol {N,N'-Bis [2-hydroxy-1-(hydroxymethyl)ethyl]-5-[(2S)-2-hydroxypropanoylamino]-2,4,6-triiodoisophthalamide} (total amount, 100 mL; injection rate, 3 mL/s), the lesion was well enhanced in the arterial phase (Figure 1B) and showed washout in the equilibrium phase (Figure 1C). Although the tumor margin was irregular without capsule-like enhancement, the internal enhancement pattern was consistent with classical HCC.

On MRI, the tumor was hypointense on T1-weighted imaging and hyperintense on T2-weighted imaging relative to the liver parenchyma (Figure 2A). Diffusion-weighted images showed that the lesions had very high intensity (Figure 2B). Opposed-phase images showed no signal intensity reduction compared with in-phase images (Figure 2C and D), indicating no fatty component in the tumor. The MRI were also consistent with HCC.

This tumor was diagnosed as an HCC from the imaging studies, and hepatectomy was performed. Based on the findings of intraoperative ultrasonography, the tumor was located close to the right hepatic vein, and subsegmental resection combined with the hepatic vein was performed. Macroscopically, the cut surface of the surgical specimen showed a yellowish-white, solid tumor, measuring 1.5 cm × 2.2 cm. Although the tumor margin was clear, there was no fibrous capsule. Neither hemorrhage nor large blood vessels were observed in the tumor. The outline margin with the surrounding liver was irregular, indicating cholangiocarcinoma (Figure 3).

Pathologically, cuboidal to columnar cells with copious clear cytoplasm proliferated in the ductal structures (Figure 4A). Most of the tumor showed a well-differentiated glandular pattern (Figure 4B), and there was an area showing a poorly differentiated component without conspicuous lumens and forming solid nests (Figure 4C).

The tumor cells showed a weak reaction to periodic acid Schiff (PAS) and were digested by diastase treatment (Figure 5A and B). Neither mucicarmine nor Alcian blue stained the tumor cells (Figure 5C and D).

Immunohistochemical staining showed positive reactions for cytokeratin (CK) 7, CK19, vimentin (cell membranes), CD56 (focally), and epithelial membrane antigen (EMA) (membranes of the luminal side) (Figure 6), and negative reactions for CK20, AFP, hepatocyte, and carcinoembryonic antigen (CEA) (Table 3).

Based on the histopathologic and immunohistochemical findings, the tumor was diagnosed as intrahepatic cholangiocarcinoma, clear cell variant. In the liver parenchyma, mild fatty change of hepatocytes, mild fibrosis and bridging of portal areas, and inflammatory infiltrates were observed.

Postoperatively, the patient was given adjuvant chemotherapy with tegafur-gimeracil-oteracil potassium {5-fluoro-1-[(2*RS*)-tetrahydrofuran-2-yl] uracil-5-chloro-2,4-dihydroxypyridine-monopotassium1,2,3,4-tetrahydro-2,4-dioxo-1,3,5-triazine-6-carboxylate} and is presently, 7 mo after surgery, free from tumor recurrence.

DISCUSSION

ICC is an epithelial malignancy that originates in the intrahepatic bile duct epithelium. Most ICCs are adenocarcinomas showing a tubular pattern, while some show cords or papillary structures[1]. Variants include adenosquamous carcinoma, sarcomatoid carcinoma, mucinous carcinoma, mucoepidermoid carcinoma, signet-ring cell carcinoma, and squamous carcinoma[1,4-6]. There is also a very rare variant, the clear cell variant[1,4-11].

Only 8 cases of the clear cell variant of ICC have been reported until now[4,7-11]. As the number of cases is very low, there is not a clear definition of histology and background disease. The reported background diseases were one case with diabetes mellitus and hypertension[8] and 2 cases with cholecystectomy[4] in the previous 8 reported cases. The present case had chronic hepatitis B as the background disease, that had not been reported.

The clear cell variant of ICC is characterized by tubular structures that consist of clear cells with copious clear cytoplasm, as well as a solid nest pattern[10] and papillary

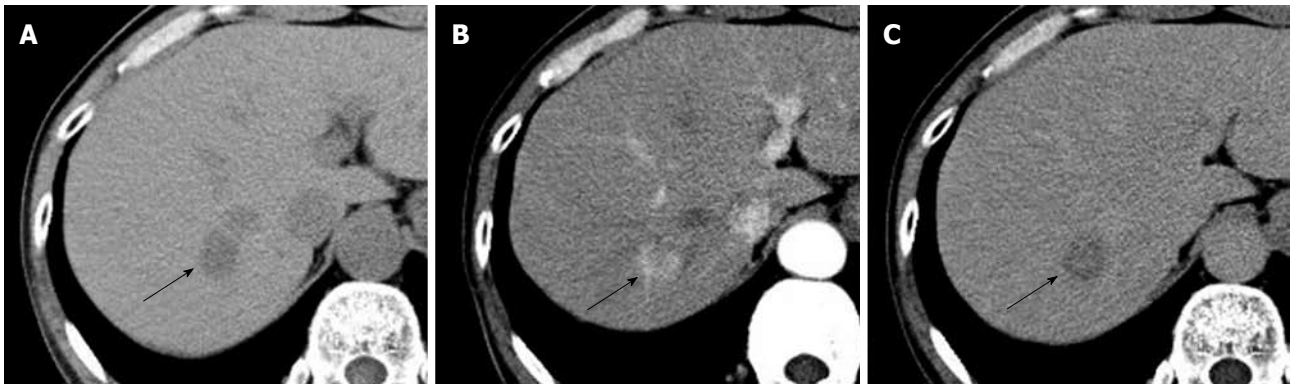


Figure 1 Findings of contrast enhanced computed tomography (CT) of the abdomen (arrows indicate the tumor). A: Plain CT shows a well-circumscribed mass in the posterosuperior S7 of the right lobe, measuring approximately 2 cm in diameter, with no calcification or fatty component; B: Enhanced CT of the arterial phase shows that it is well-enhanced; C: Enhanced CT of the equilibrium phase shows a washed-out image.

Table 1 Reported cases of intrahepatic cholangiocarcinoma clear cell variant

Yr	Age (yr)/sex	Location	Size (cm)	Hepatitis	Liver cirrhosis	Mechanism	Prognosis
1998[10]	64/F	Unknown	12	Unknown	Unknown	Unknown	Unknown
1998[11]	62/M	Right lobe	5	-	-	Unknown	Dead, 14 mo; no treatment
1998[7]	72/M	Left lobe	15	Unknown	Unknown	Lipid >> glycogen	Alive, 30 mo
1999[8]	50/M	S6	1.5	-	Unknown	Mucin	Alive, 12 mo
2001[9]	64/M	Right lobe	6	-	-	Unknown	Alive, 3 yr
2007[4]	51/M	S4a	9	Unknown	-	Lipid >> glycogen	Meta, 1 yr; dead, 3 yr
2007[4]	60/F	S5	4.5	Unknown	-	Lipid >> glycogen	Alive, 1 yr
2007[4]	58/F	S5	2.3	Unknown	-	Lipid >> glycogen	Alive
Present case	56/M	S7	2.2	HBV	-	Glycogen	Alive, 7 mo

M: Male; F: Female; S: Segment; meta: Metastasis.

Table 2 Serum data on admission

Item	Data	Normal values
HBs-Ag (C.O.I)	277.5	< 1.0
HBs-Ab (mIU/mL)	165.1	< 5.0
Anti-HCV-Ab (C.O.I)	0.1	< 1.0
α-fetoprotein (ng/mL)	3.6	≤ 7.0
PIVKA-Ⅱ (mAU/mL)	17.0	< 40.0
Carcinoembryonic antigen (ng/mL)	2.4	≤ 5.0
Total cholesterol (mg/dL)	181.0	128-25
Aspartate aminotransferase (IU/L)	28.0	13-33
Alanine aminotransferase (IU/L)	21.0	8-42
Alkaline phosphatase (IU/L)	198.0	115-359
Lactate dehydrogenase (IU/L)	207.0	119-229
γ-glutamyl transpeptidase (IU/L)	30.0	10-47

HBs-Ag: Hepatitis B surface antigen; HBs-Ab: Hepatitis B surface antibody; anti HCV-Ab: Anti hepatitis C virus antibody; PIVKA: Protein induced by vitamin K absence; C.O.I: Cut off index.

Table 3 Immunohistochemical staining

Antibody	Company	Result
CK7	DAKO	+
CK19	DAKO	+
CK20	DAKO	-
CAM5.2	Becton, Dickinson, and Co.	+
EMA	DAKO	+
CA19-9	DAKO	+ focal
CEA	DAKO	-
CD56	DAKO	+ focal
AE1/3	DAKO	+
Ki-67 (MIB-1)	Novocastra	7.5%[1]
p53	Novocastra	+ focal
Bcl-2	DAKO	-
TTF-1	DAKO	-
Hepatocyte	DAKO	-
a-Fetoprotein	DAKO	-
Vimentin	DAKO	+
CD10	DAKO	-
S-100 protein	DAKO	-
Chromogranin	DAKO	-
Synaptophysin	DAKO	-

[1]Ki-67(MIB-1) index was estimated at the most frequent area. CAM: Cell adhesion molecule; CEA: Carcinoembryonic antigen; TTF-1: Thyroid transcription factor-1; CD: Cluster of differentiation.

pattern[9]. The neoplastic cells are medium sized with a vacuolated or clear cytoplasm and have distinct cell borders[4,7]. The nuclei of the clear cells are round to ovoid and hyperchromatic[9]. In the present case, most of the tumor showed a well-differentiated glandular pattern, though there were some areas showing a poorly differentiated component without conspicuous lumens and forming solid nests. The cells were of medium size in the glandular areas and were smaller in the solid areas.

The cause of the clear cell change is said to be glycogen, based on electron microscopy and diastase diges-

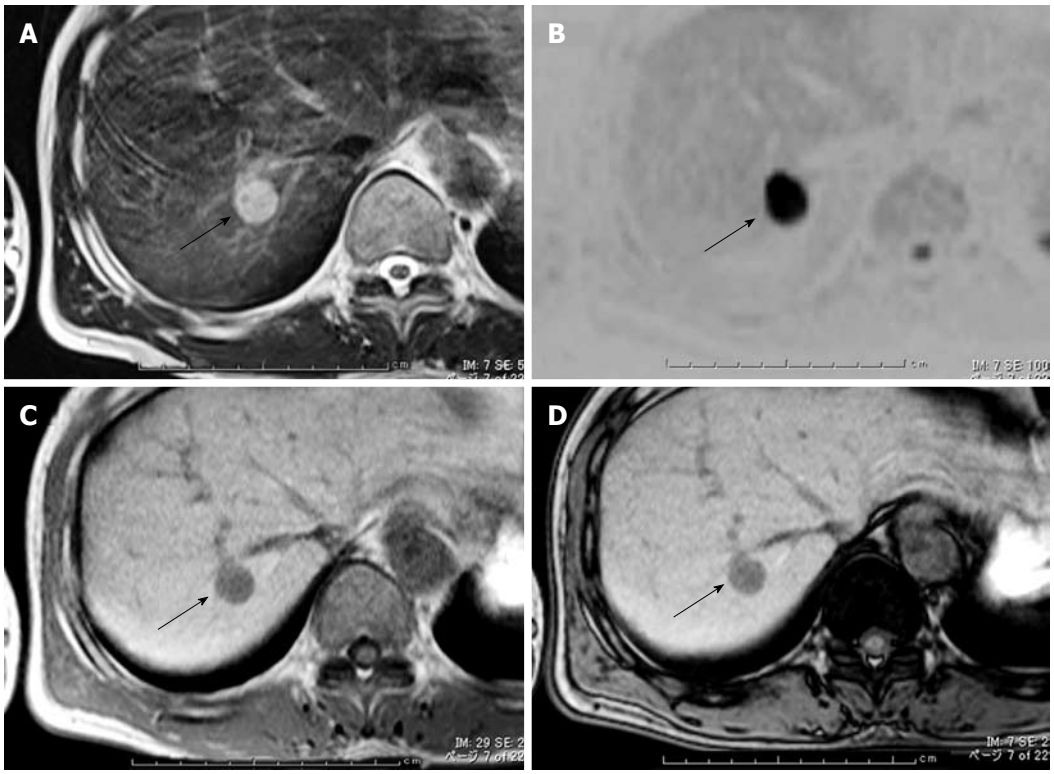


Figure 2 Findings of magnetic resonance imaging (arrows indicate the tumor). A: The T2-weighted image shows a hyperintense tumor; B: The inverse video of the diffusion-weighted image shows a markedly hyperintense signal; C: In-phase T1-weighted image shows a hypointense signal; D: Opposed-phase T1-weighted image shows no signal intensity reduction compared with Figure 2C.

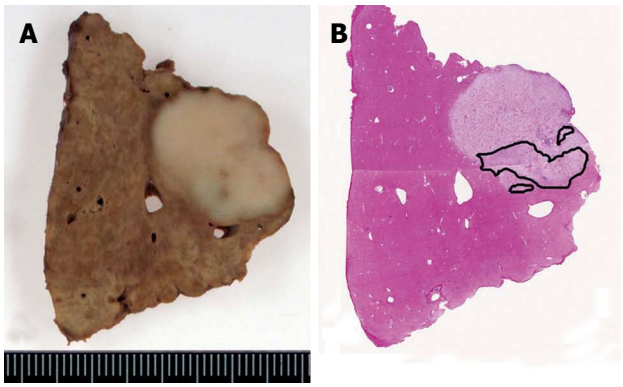


Figure 3 Tumor images. A: Photograph of the cut surface of the tumor fixed in formalin shows a yellowish-white, solid tumor, measuring 1.5 cm × 2.2 cm. Although the tumor margin is clear, there is no fibrous capsule. Neither bleeding nor blood vessels inside the tumor are observed. The outline margin with the surrounding liver is irregular; B: Scanning image. The tumor consists of clear cells. The margin is clear. The area surrounded by the black line is the poorly differentiated area.

tion[4,7], mucin, based on positive staining with either mucicarmine or Alcian blue[4,7,9], and lipid, based on electron microscopy[4,7]. These cases may represent the same disease entity only because they show similar clear changes, despite having different causes. In the present case, mucin did not stain, the radiological findings did not support a fatty component[12], and only PAS with diastase suggested glycogen.

Immunohistochemically, tumor cells were CK7-positive[4,7-9,11] and CK20-negative[4,7-9], and both HepPar1[4,9] and AFP-negative[4,7,8,11]. CD56-positivity was reported recently by Haas *et al*[4], who argued that the reactivity was related to the clear cell change. However, ICCs with hepatitis B or C were also reported to be CD56-positive[13,14]. Therefore, it is uncertain whether the background is responsible for CD56 reactivity. As CD56 had not been determined in the reports before Haas *et al*[4], the reactivity for CD56 may be a new feature of clear cell ICC. The expression of vimentin usually relates to mesenchymal cells, though some epithelial tumors such as renal cell carcinoma (RCC), some ICCs[14], and some clear cell ICCs were seen in previous[4] cases and in the present case.

The clear cell variant of ICC should be distinguished from clear cell HCC and metastatic clear cell carcinomas arising from extrahepatic organs, especially RCC. Positivity for CK7 and negativity for CK20 correspond to cholangiocarcinoma[15]. Hepatocyte negativity, AFP-negativity, and formation of tubular structures can rule out HCC[4,7-9,11]. Lack of detection of mucin and small gland formation may be similar to cholangiolocellular carcinoma. Cholangiolocellular carcinomas can be differentiated in that they usually show numerous nodules composed of cells smaller than normal hepatocytes[16,17] and are negative for vimentin and CD56[14]. The differential diagnosis from RCC is achieved by detailed examination of the kidneys and negative staining for vimentin, CD10, and S-100 protein[4,7,9,10]. The present case was CD10- and S-100 protein-negative and vimentin-positive, but reactivity for vimentin

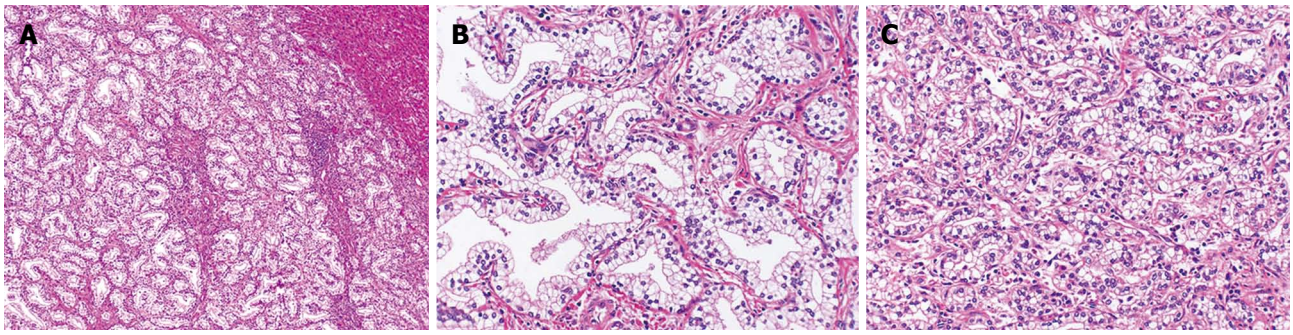


Figure 4 Pathological findings (hematoxylin-eosin). A: Clear tumor cells proliferate in a tubular shape. The pushing margin is irregular (× 40); B: Area of well-differentiated duct formation. The tumor cells have copious clear cytoplasm (× 200); C: The poorly differentiated area shows solid proliferation (× 200).

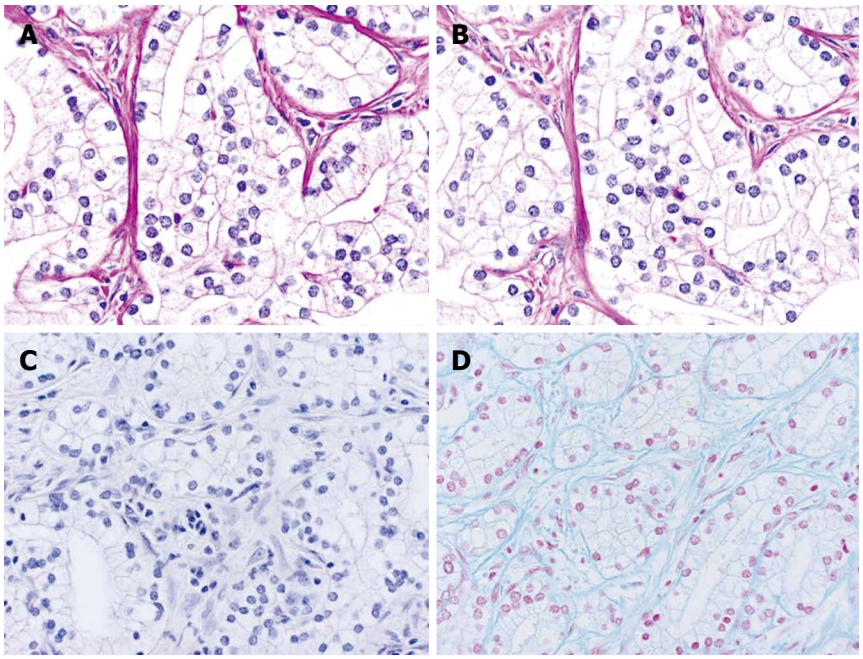


Figure 5 Particular stains (× 400). A: The tumor cells show a weak reaction for periodic acid Schiff (PAS) stain; B: PAS stain is slightly digested by diastase treatment; C: Mucicarmine is negative; D: Alcian blue is negative.

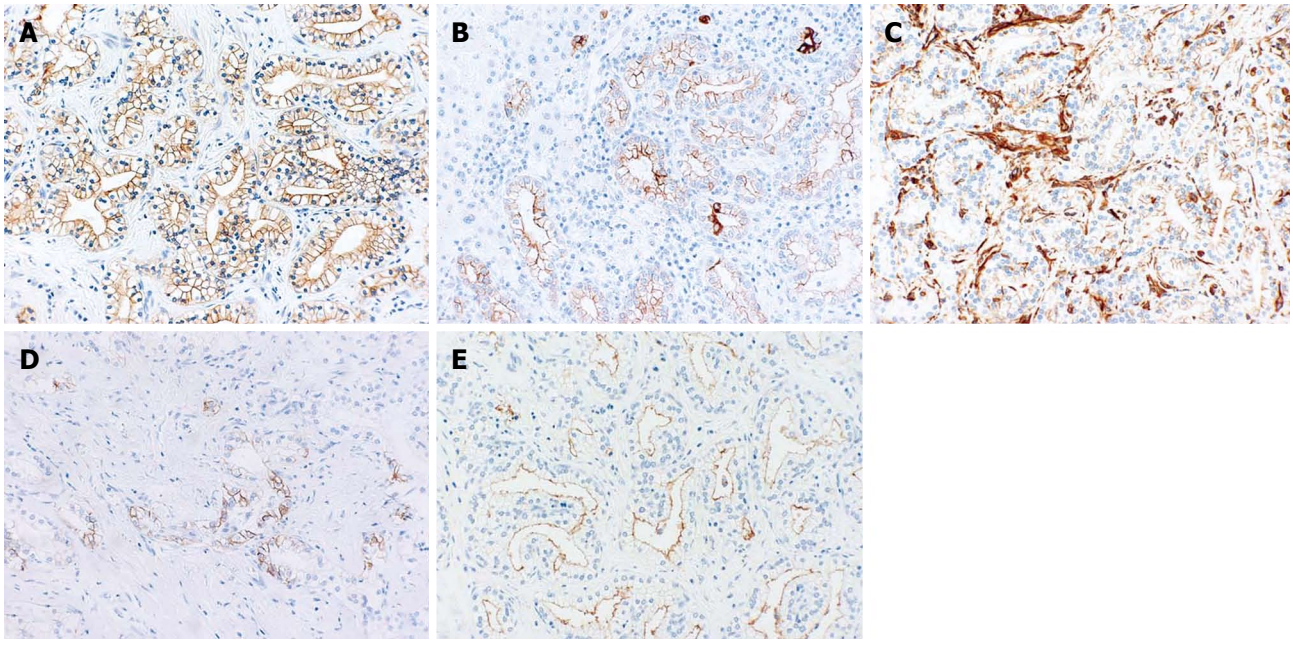


Figure 6 Immunohistochemical staining (× 200). A: Cytokeratin (CK) 7 is positive; B: CK19 is positive; C: Vimentin is positive; D: CD56 is focally positive; E: Epithelial membrane antigen (EMA) is positive at the membrane of the luminal side.

in ICC has also been reported[4,14]. In addition, there were no abnormalities in the kidneys in careful radiological examination. Clear cell carcinoma of the gastrointestinal tract can be differentiated by detailed examination and histopathological findings.

Benign clear cell tumors of the bile ducts have seldom been reported except for 3 cases of atypical bile duct adenoma, clear cell type[9]. Immunohistochemically, they showed similar reactivity to their malignant counterpart, i.e. positivity for CK7, CEA, EMA and p53 and negativity for CK20. The only reported differential point was positivity rate of Ki-67 (MIB-1) of under 10% for bile duct adenomas and of over 50% for ICCs[9]. However, the ICC clear cell variant has also been reported to show positivity of less than 5%[4]. Thus, the conclusion has not been ascertained. Bile duct adenoma is said to be under 1 cm in diameter, located under the capsules and have less irregularity of the nuclei[1]. The present case showed Ki-67 positivity of under 10%. However, the size was 2.2 cm and it was located deep in the parenchyma apart from the capsule, with the nuclei showing irregularity, indicating a diagnosis of carcinoma.

ICC is said to have a poor prognosis[2,14] because of its tendency to be diagnosed at a late stage and the absence of effective treatment[3]. While the ICC clear cell variant has been reported to have a good prognosis[4,7-9], except for one death with metastasis after hepatic resection[4], and one case that had not been operated on because of underlying diseases and died 14 mo later[11]. In this case, the patient is currently free from disease, and the medication for chronic hepatitis B and the postoperative chemotherapy has been continued. Because the number of cases is very low, there is no evidence for the use of chemotherapy. Therefore, careful follow-up with sufficient postoperative supplementary chemotherapy is necessary to achieve longer survival.

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Mourning for renowned pharmacologist Professor Geng-Tao Liu

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Figure 1 Geng-Tao Liu, Professor, Department of Pharmacology, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050, China

At 22:08 h on February 27, 2010, Professor Geng-Tao Liu, a renowned pharmacologist affiliated with the Department of Pharmacology, Institute of Materia Medica, Chinese Academy of Medical Sciences, Beijing, an academician of the Chinese Academy of Engineering, a doctoral tutor, and a member of *World Journal of Gastroenterology* (*WJG*) Editorial Board, passed away at Peking Union Medical College Hospital, at the age of 77 years (Figure 1).

Professor Liu was born in 1932 in Shuangfeng, Hunan Province. He graduated from Hunan Medical College in 1956 and then started his research career in the Department of Pharmacology, Institute of Materia Medica, Chinese Academy of Medical Sciences. He was elected a member of the 6-8th Council of Chinese Pharmacological Society.

During his research career, Professor Liu successfully developed two novel anti-hepatitis drugs: dimethyl diphenyl bicarboxylate and bicyclol. He also made great contributions to the understanding of the pharmacology of *Schisandra*, *Ganoderma lucidum,* and *Salvia miltiorrhiza*.

Professor Liu successfully developed Ganoderma Spore Powder Injection that has been used in China for over 30 years to treat a variety of neuromuscular ailments. To evaluate the pharmacological effects of Ganoderma lucidum, Professor Liu created two unique animal models: one for dermatomyositis and the other for elevated serum aldolase, which provide a basis for a scientific explanation of the therapeutic effects of Ganoderma lucidum against dermatomyositis. He also found that several schizandrins, particularly schisanhenol, have potent antioxidant activity.

Professor Liu published over 200 papers and was granted multiple patents. He is the editor-in-chief of "*Contemporary Pharmacology*" and the editor of "*Pharmacology of Traditional Chinese Herbs and Drug Innovation*". He was a member of the editorial board of 12 domestic and foreign academic journals. He was awarded nine scientific or technological prizes in China, such as "National Invention Award", "Scientific and Technological Progress Award of the Ministry of Health", and "Beijing Municipal Scientific and Technological Progress Award". Professor Liu also won the titles of "National Young and Middle-aged Experts with Outstanding Contributions",

"Capital May 1st Labor Medal", and "Beijing Municipal Outstanding Teacher". Having tutored more than 40 postgraduates, Professor Liu enjoyed a great reputation in the field of pharmacology.

Professor Liu took time out of his busy schedule to review six *WJG* articles and made a great contribution to *WJG*. Now, he has left us forever! We are deeply saddened by his passing and will turn our grief into strength to continue our efforts to establish *WJG* as one of the leading journals. Professor Liu, may you rest in peace.

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Meetings

Events Calendar 2010

January 25-26
Tamilnadu, India
International Conference on Medical Negligence and Litigation in Medical Practice

January 25-29
Waikoloa, HI, United States
Selected Topics in Internal Medicine

January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology Conference

January 28-30
Hong Kong, China
The 1st International Congress on Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of Gastroenterology & Endoscopy Meeting

March 09-12
Brussels, Belgium
30th International Symposium on Intensive Care and Emergency Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian National Association for Study of the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on Diabetes PACD14

March 25-28
Beijing, China
The 20th Conference of the Asian Pacific Association for the Study of the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology and Hepatology Conference, EGHC 2010

April 14-17
Landover, Maryland, United States
12th World Congress of Endoscopic Surgery

April 14-18
Vienna, Austria
The International Liver Congress™ 2010

April 28-May 01
Dubrovnik, Croatia
3rd Central European Congress of surgery and the 5th Croatian Congress of Surgery

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual Meeting

May 06-08
Munich, Germany
The Power of Programming: International Conference on Developmental Origins of Health and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and Rectal Surgeons Annual Meeting

June 04-06
Chicago, IL, United States
American Society of Clinical Oncologists Annual Meeting

June 09-12
Singapore, Singapore
13th International Conference on Emergency Medicine

June 14
Kosice, Slovakia
Gastro-intestinal Models in the Research of Probiotics and Prebiotics-Scientific Symposium

June 16-19
Hong Kong, China
ILTS: International Liver Transplantation Society ILTS Annual International Congress

June 20-23
Mannheim, Germany
16th World Congress for Bronchoesophagology-WCBE

June 25-29
Orlando, FL, United States
70th ADA Diabetes Scientific Sessions

August 28-31
Boston, Massachusetts, United States
10th OESO World Congress on Diseases of the Oesophagus 2010

September 10-12
Montreal, Canada
International Liver Association's Fourth Annual Conference

September 11-12
La Jolla, CA, United States
New Advances in Inflammatory Bowel Disease

September 12-15
Boston, MA, United States
ICAAC: Interscience Conference on Antimicrobial Agents and Chemotherapy Annual Meeting

September 16-18
Prague, Czech Republic
Prague Hepatology Meeting 2010

September 23-26
Prague, Czech Republic
The 1st World Congress on Controversies in Gastroenterology & Liver Diseases

October 07-09
Belgrade, Serbia
The 7th Biannual International Symposium of Society of Coloproctology

October 15-20
San Antonio, TX, United States
ACG 2010: American College of Gastroenterology Annual Scienitfic Meeting

October 23-27
Barcelona, Spain
18th United European Gastroenterology Week

October 29-November 02
Boston, Massachusetts, United States
The Liver Meeting® 2010--AASLD's 61st Annual Meeting

November 13-14
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Case-Based Approach to the Management of Inflammatory Bowel Disease

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The biggest advantage of the OA model is that it provides free, full-text articles in PDF and other formats for experts and the public without registration, which eliminates the obstacle that traditional journals possess and usually delays the speed of the propagation and communication of scientific research results. The open access model has been proven to be a true approach that may achieve the ultimate goal of the journals, i.e. the maximization of the value to the readers, authors and society.

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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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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/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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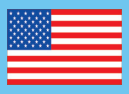
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EDITORIAL

Soft drinks consumption and nonalcoholic fatty liver disease

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is a common clinical condition which is associated with metabolic syndrome in 70% of cases. Inappropriate dietary fat intake, excessive intake of soft drinks, insulin resistance and increased oxidative stress combine to increase free fatty acid delivery to the liver, and increased hepatic triglyceride accumulation contributes to fatty liver. Regular soft drinks have high fructose corn syrup which contains basic sugar building blocks, fructose 55% and glucose 45%. Soft drinks are the leading source of added sugar worldwide, and have been linked to obesity, diabetes, and metabolic syndrome. The consumption of soft drinks can increase the prevalence of NAFLD independently of metabolic syndrome. During regular soft drinks consumption, fat accumulates in the liver by the primary effect of fructose which increases lipogenesis, and in the case of diet soft drinks, by the additional contribution of aspartame sweetener and caramel colorant which are rich in advanced glycation end products that potentially increase insulin resistance and inflammation. This review emphasizes some hard facts about soft drinks, reviews fructose metabolism, and explains how fructose contributes to the development of obesity, diabetes, metabolic syndrome, and NAFLD.

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Key words: Aspartame; Caramel; Carbonated beverage; Cola; Diabetes; Fatty liver; Fructose; Metabolic syndrome; Obesity; Soda; Soft drink; Sweetened beverage

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a significant health problem affecting 20%-30% of the adult population[1]. NAFLD can progress to nonalcoholic steatohepatitis (NASH), a fatty liver with hepatitis. This form of liver injury carries a 20%-50% risk for progressive fibrosis, 30% risk for cirrhosis, and 5% risk for hepato- cellular carcinoma[2-4]. Although the mechanisms underlying dis-

ease progression remain unclear, insulin resistance and obesity-related inflammation are thought to play a key role, along with possible genetic, dietary and lifestyle factors. The rising incidence of obesity in today's generation is associated with many health complications in addition to NAFLD[5,6]. These include cardiovascular diseases, diabetes, hyperlipidemia, and hypertension. This constellation is recognized as metabolic syndrome. 70% of patients with fatty liver have metabolic syndrome and 30% of patients with metabolic syndrome have fatty liver[7] (Figure 1).

A global change in dietary habits has occurred over the last few decades resulting from the introduction of sweeteners such as fructose and sucrose by the food industries. For example, regular soft drinks (SD) and fruit drinks, major sources of high fructose corn syrup (HFCS) or sugar, have increased from 3.9% of the total energy intake in 1977 to 9.2% of the total energy intake in 2001[8].

Worldwide, SD are the leading cause of added sugar. Recent evidence suggests an association between the intake of sugar sweetened SD and the risk of obesity and diabetes resulting from large amounts of HFCS used in their manufacture, which raises blood glucose similar to sucrose[9]. In addition, diet SD contain aspartame sweetener and caramel coloring, which are rich in advanced glycation end products that potentially increase insulin resistance and inflammation[10,11].

Human studies and animal models suggest that dietary factors can affect fatty infiltration and lipid peroxidation in various types of liver disease including NAFLD[12,13]. More recently, increased ingestion of SD was found to be linked to NAFLD[14] independent of metabolic syndrome, with NAFLD patients consuming 5 times the amount of carbohydrates from SD as compared to healthy persons[15] (Figure 2 and Table 1). Individuals consuming > 1 soft drink daily showed a higher prevalence of metabolic syndrome than those consuming < 1 soft drink per day[16].

This review emphasizes some hard facts about SD, reviews fructose metabolism, and explains how fructose contributes to the development of obesity, diabetes, metabolic syndrome, and NAFLD.

SOFT DRINKS

The term SD more commonly known as soda, soda pop, pop, Coke™, Pepsi™ or tonic, refers to a nonalcoholic beverage that is usually carbonated. Two types of SD are used; regular SD which are sweetened with sugar (fructose) and diet SD which are sweetened with non-caloric sweeteners (aspartame). Up to the 1980s, SD contained most of their food energy in the form of refined cane sugar or corn syrup. Today, HFCS is used almost exclusively as a sweetener in the United States and in other countries because of its lower cost. The calories and sugar content in various soft drinks are shown in Table 2.

Added sweeteners in regular SD are an important component of our diet, representing 318 kcal of dietary intake, or 16% of all calorie intake[17]. HFCS made by enzymatic isomerization of glucose to fructose was introduced as HFCS-42 (42% fructose) and HFCS-55 (55% fructose) in 1967 and 1977, respectively, and opened a new frontier for the sweetener and SD industries.

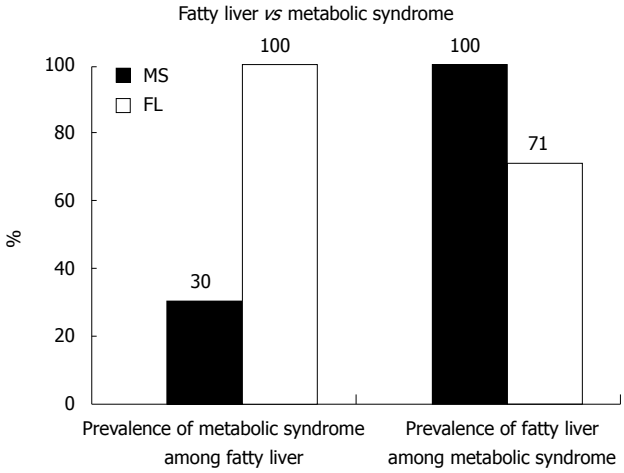


Figure 1 Prevalence of fatty liver among metabolic syndrome and prevalence of metabolic syndrome among fatty liver. MS: Metabolic syndrome; FL: Fatty liver. Alberti, Circulation (2009). $P < 0.001$.

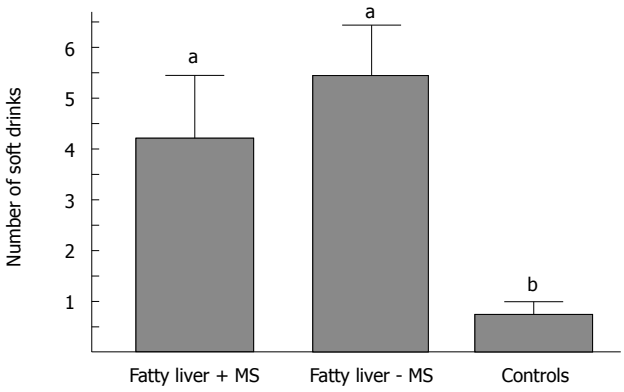


Figure 2 Daily amount of soft drinks consumption in nonalcoholic fatty liver disease (NAFLD) patients with (n = 31) or without metabolic syndrome (n = 29) and in controls (n = 30). [a]$P < 0.07$; fatty liver without metabolic syndrome *vs* fatty liver with metabolic syndrome, [b]$P < 0.001$ between fatty liver subgroups and controls[14,15].

Table 1 Soft drink consumption linked with NAFLD

Dietary constituents	Controls (n = 30)	NAFLD (n = 31)	P value
Total energy intake (kcal)	2200 ± 600	2300 ± 500	0.300
Added sugar (g/d)	33.6 ± 12.6	75.6 ± 8.4	0.001
Percent of added sugar from soft drinks	8%	43%	0.001

NAFLD: Nonalcoholic fatty liver disease.

Aspartame and caramel (colorant) are also used as sweeteners in the beverage industry mainly in diet SD[18]. Aspartame is an amino-acid compound that is about 160 times sweeter than sugar. Aspartame is absorbed from the intestine and metabolized by the liver to form phenylalanine, aspartic acid and methanol. Aspartame can contribute to weight gain, obesity, insulin resistance, and type 2

Table 2 Calories and sugar content in different soft drinks

Soft drinks: calorie content (number of calories)				Soft drinks: sugar content (numbers of teaspoons of sugar)			
	12- oz. Can	20 oz. Bottle	64 oz. Big cup		12- oz. Can	20 oz. Bottle	64 oz. Big cup
Sunkist	190	325	1040	Orange slice	11.9	19.8	63.5
Mountain dew	165	275	880	Mint maid orange soda	11.2	18.7	59.7
Dr. Pepper	160	250	800	Mountain dew	11.0	18.3	58.7
Pepsi	150	250	800	Barq's root beer	10.7	17.8	57.1
Coke classic	140	250	800	Pepsi	9.8	16.3	52.3
Sprite	140	250	800	Squirt	9.5	15.8	50.7
7-Up	140	250	800	Dr. Pepper	9.5	15.8	50.7
				7-Up	9.3	15.5	49.6
				Coke classic	9.3	15.5	49.6
				Sprite	9.0	15.0	48.0

diabetes mellitus[18]. Recently, Brown *et al*[19] showed that artificial sweeteners may trigger the secretion of glucagon-like peptide (GLP)-1 by the digestive tract, and thereby curb appetite and calorie intake.

Caramel is made by the carefully controlled heat treatment of carbohydrates, generally in the presence of acids and alkalis, in a process called caramelization. Soft drinks contain caramel coloring, which is rich in advanced glycation end products which increase insulin resistance and inflammation[9,10]. The FDA has established 200 mg of caramel per kg body weight as an acceptable daily intake.

High fructose diets have induced fatty liver in rats and ducks[20]. Such diets have also caused increases in hepatic lipid peroxidation and activation of inflammatory pathways in the liver of rats[21]. The inborn error of metabolism known as hereditary fructose intolerance, a rare disease which results from a deficiency in the fructose metabolizing enzyme, aldolase B, has demonstrated that fructose consumption can cause progressive liver disease in humans[22].

The extent to which excessive fructose might contribute to the high prevalence of NAFLD in Western societies has not been systematically investigated. It has been shown that consumption of SD is linked to obesity and results in an increased risk of metabolic syndrome. Individuals consuming > 1 soft drink per day had a higher prevalence of metabolic syndrome than those consuming < 1 drink per day[16].

METABOLISM OF FRUCTOSE

Fructose is a simple sugar with a chemical formula ($C_6H_{12}O_6$) similar to that of glucose. Fructose differs from glucose by the presence of a keto group attached to carbon 2 of the molecule, while glucose has an aldehyde group at carbon 1. In the diet, fructose is consumed in various amounts with fruits, honey, beverages sweetened with HFCS/sucrose and as a constituent of sucrose, the most common sugar (a disaccharide composed of fructose through a 1-4 glycoside bond) (Table 2).

Absorption of fructose from the intestine into the portal blood is aided by glucose transporter-5 at the brush border and basolateral membranes of the jejunum. This route of absorption results in massive fructose uptake by the liver. Fructose is phosphorylated by fructokinase, forming fructose-1-phosphate, which can then be converted to several three-carbon molecules, including glyceraldehydes, dihydroxyacetone phosphate and glyceraldehyde-3-phosphate (Figure 3). Some of these 3 carbon molecules can be converted to glucose through gluconeogenesis, or they can be used to generate other products such as triglyceride (TG).

The second metabolism of fructose, i.e. the extrahepatic metabolism that bypasses fructokinase, allows the carbons from fructose to enter glycolysis downstream of this enzyme. The 3 carbon molecules can eventually be used for the synthesis of glycerol and fatty acids, which through esterification can form TGs.

The concentration of fructose in fasting blood of healthy humans is typically 1 mg/dL or less. After oral administration of fructose load in doses ranging from approximately 18 g (0.25 g/kg of body weight) to 100 g, the mean plasma or serum fructose concentration increased in a dose-dependant manner, to values ranging from 4.5-13.0 mg/dL and peak fructose concentrations were seen 30-60 min after fructose ingestion. A 20-ounce soft drink containing 32.6 g of fructose would therefore be expected to increase the fasting serum fructose concentration by approximately four-fold[23,24]. Fructose is 7 times more likely than glucose to form advanced glycation end products (AGEs). Fructose does not suppress ghrelin and does not stimulate insulin or leptin[23,24]. Some key molecular features involved in the metabolism of fructose include the roles of cellular signaling molecules including nuclear factor-κB (NF-κB), tumor necrosis factor-α (TNF-α), c-Jun amino terminal kinase 1 (JNK-1), protein tyrosine phosphatase-1B (PTP-1B), phosphatase and tensin homolog deleted on chromosome ten (PTEN), liver X receptor (LXR), farnesoid X receptor (FXR), and sterol regulatory element-binding protein-1c (SREBP-1c)[25]. Fructose activates JNK-1, which causes hepatic inflammation and increased insulin receptor substrate-1 (IRS-1). Fructose induces lipogenesis *via* upregulation of SREBP-1c and CHREBP, thereby increasing the hepatic pool of free fatty acids[25].

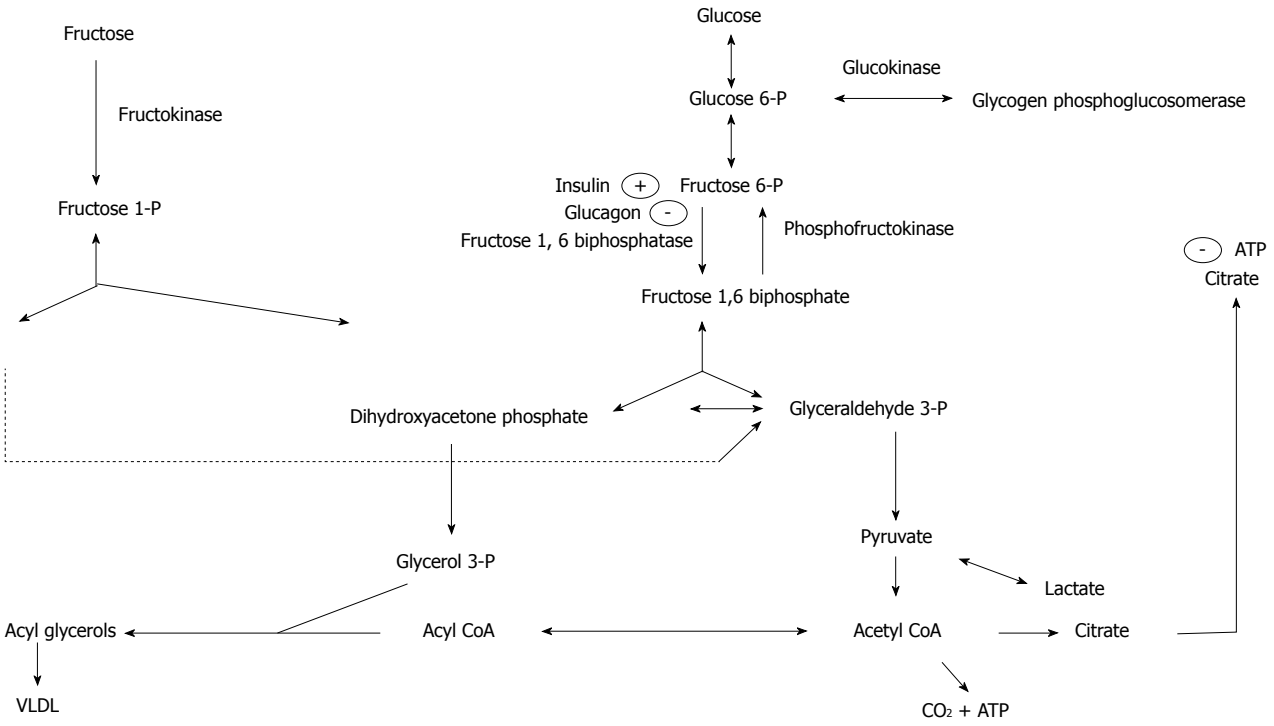


Figure 3 Fructose metabolism in the liver. Hepatic fructose metabolism begins with phosphorylation of fructokinase. Fructose carbon enters the glycolytic pathway at the triose phosphate level. Thus, fructose bypasses the major control point by which glucose carbon enters glycolysis. This allows fructose to serve as an unregulated source of glycerol-3-phosphate and acetyl-CoA for hepatic lipogenesis.

PATHOPHYSIOLOGY OF NAFLD

The "two hits" hypothesis proposed by Day *et al*[26] remains the prevailing pathophysiological theory. According to the authors, the first "hit" describes a net retention of lipids within hepatocytes, mostly in the form of TGs, and is a prerequisite for the development of NAFLD. A continuous delivery of free fatty acids to the liver from splanchnic lipolysis of visceral fat (60%) or from increased ingestion of fatty food (10%), combined with peripheral insulin resistance, and *de novo* lipogenesis (30%) results in excessive fat accumulation and an increased liver concentration of TG and cholesterol esters. High blood TG concentration in the form of very low density lipoprotein (VLDL) tends to accompany this condition and induces cholesterol ester transfer protein activity, resulting in an increased transfer of TG from VLDL to high density lipoprotein (HDL) and a subsequent increase in HDL clearance and decreased HDL concentration which leads in the end to liver steatosis[27].

The progression of steatosis to steatohepatitis (NASH) is associated with other factors ("second hit"), such as lipotoxicity, inflammation, oxidative stress and insulin resistance[26]. Consumption of SD may act as a first or as a second hit in the pathogenesis of NAFLD. Recently, it has been suggested that cholesterol metabolism may have a role in the accumulation of liver fat and that inflammation may be the first hit followed by TG accumulation as a second hit (www.easl.eu/bologna 2009).

FRUCTOSE AND INSULIN RESISTANCE

Fructose consumption increases postprandial TG concentrations within 24 h[28,29], which suggests that postprandial hypertriglyceridemia is the earliest metabolic perturbation associated with fructose consumption. The most likely mechanism for postprandial hypertriglyceridemia is increased hepatic de-novo lipogenesis (DNL), which in turn upregulates VLDL production and secretion[30].

Fructose consumption can promote hepatic lipogenesis primarily because the liver is the main site of fructose metabolism; secondly, entry of fructose into glycolysis *via* fructose-1-phosphate bypasses the main rate controlling step of glycolysis catalyzed by phosphofructokinase, thus providing unregulated amounts of the lipogenic substrates acetyl-CoA and glycerol-3-phosphate[30]; thirdly, fructose can activate sterol receptor element binding protein-1c (SREBP-1c) independently of insulin, which then activates fat genes involved in DNL[31,32].

Recently, Stanphone demonstrated that consuming fructose-sweetened beverages, not glucose-sweetened beverages increases DNL, promotes dyslipidemia, decreases insulin sensitivity and increases visceral adiposity in overweight and obese adults[33,34] (Figure 4).

One study of lean women found that 4 d of over feeding with a sucrose-sweetened (glucose + fructose) drink increased DNL by 200%-300%[35]. Another feeding study showed that 2 d of high fructose intake (30% of kcal/d, consumed as sweetened beverage at every meal)

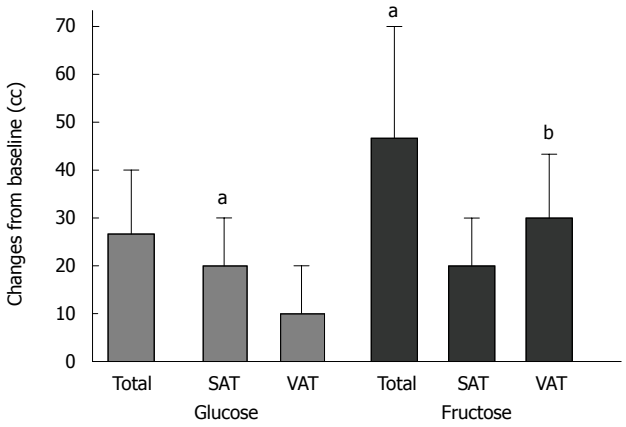


Figure 4 Changes in total abdominal adipose tissue, superficial adipose tissues (SAT), and visceral adipose tissue (VAT) volume after consuming glucose- or fructose-sweetened beverages for 10 wk. $^{a}P < 0.05$, $^{b}P < 0.01$, 10 wk *vs* 0 wk; paired Student's *t* test. Glucose, $n = 14$; Fructose, $n = 17$. Data represent mean ± SE (Stanhope, *J Clin Invest*, 2009).

resulted in decreased postprandial glucose concentration and insulin response and prolonged alimentary lipemia in women[29]. A recent clinical study indicates that NAFLD patients have a higher intake of SD and meat and a tendency towards a lower intake of fish rich in omega-3[36].

FRUCTOSE AND DIABETES MELLITUS

Lipotoxicity can promote insulin resistance in type 3 diabetes mellitus by accelerating pancreatic β-cell failure. Elevated plasma free fatty acids are associated with the progression from pre diabetes (impaired fasting glucose IFG, impaired glucose tolerance IGT) to type 2 diabetes mellitus[37]. The presence of diabetes mellitus in patients with NASH have significant clinical implications, as NASH appears to follow a more aggressive course in the presence of hyperglycemia, placing a growing number of diabetic patients at risk of progressive liver disease[37]. In short-term studies in humans, fructose ingestion had no deleterious effect on glucose metabolism. In studies lasting 3-8 d, substitution of sucrose or starch with fructose improved glycemic control in patients with type 1 or type 2 diabetes mellitus. Similarly, no adverse effects of fructose feeding on glycemic control were seen in studies lasting 1-3 mo. In a series of patients with diet controlled type 2 diabetes, substitution of sucrose by fructose (13% of calories) for three months had no significant effect on fasting plasma glucose levels or postprandial plasma glucose and insulin responses[37].

Long-term fructose consumption may promote the development of diabetes, even though fructose usually has no adverse effects on glucose tolerance in the short- and intermediate term. In rats, long-term feeding of moderate amounts of fructose (15% of the diet by weight) resulted in impaired glucose tolerance[38], and high-fructose diets (72% by weight) resulted in the development of diabetes mellitus and diffuse glomerulosclerosis[39,40].

The association between consumption of sugar-sweetened beverages and risk of type 2 diabetes was assessed in an eight-year prospective study of 51 603 women participating in the Nurses' Health Study Ⅱ[41]. After adjustment for potential confounders, women consuming one or more sugar-sweetened soft drink daily had a relative risk (RR) of type 2 diabetes of 1.83 ($P < 0.001$) compared with those who consumed > 1 of these beverages per month. The results were attenuated after further adjustment for body mass index and caloric intake, but remained statistically significant (RR = 1.32, $P < 0.04$). Consumption of fruit punch was associated with a similar increase in diabetes risk.

FRUCTOSE AND OBESITY

The association between HFCS consumption and obesity is due in part to metabolic changes induced by fructose or HFCS, rather than merely to an increase in total energy intake[41]. In baboon studies, consumption of sucrose compared with glucose promoted the development of abdominal obesity, suggesting that the fructose moiety of sucrose was responsible for the increase in abdominal fat[42,43]. In addition, some strains of mice showed an increase in visceral fat accumulation when fed a high-fructose diet[44]. Although it has long been suspected that SD contribute at least in part to the obesity epidemic, only in recent years have large epidemiologic studies begun to investigate the relation between SD consumption and long-term weight gain. Obesity among children has increased dramatically during the past two decades and is approaching epidemic proportions[45,46]. Various environmental, genetic and social factors relating to diet have been associated with obesity in children[47-50].

The results of the study by Dubois *et al*[51] indicated that regular sugar-sweetened beverage consumption, especially between meals, may put children at greater risk for obesity in childhood. Because there is a positive association between the consumption of sugar-sweetened beverages and body weight among preschool-aged children, they advise that parents should limit the quantity of such sweetened beverages consumed during preschool years[51]. In the FIELD trial, 644 British schoolchildren (ages 7-11 years) were randomly assigned to a control group or to an education program designed to reduce their consumption of carbonated drinks (both sweetened and unsweetened). The mean consumption of carbonated drinks decreased by 50 mL/d in the intervention group and increased by 16.7 mL/d in the control group (mean difference 0.7, 95% confidence interval 0.1 to 1.3). After 12 mo, the approximate percentage of overweight and obese children had increased in the control group from 20% to 27.5%, compared with a decrease in the intervention group from 20% to 19.8% (mean difference 7.7%, 2.2% to 13.1%)[52].

FRUCTOSE AND NAFLD

Risk factors for NAFLD include obesity, type 2 diabetes, insulin resistance and hypertriglyceridemia. Of note,

each of these risk factors can occur as a result of excessive fructose consumption. Recently we have shown that[14,15] SD consumption is linked with fatty liver independently by metabolic syndrome diagnosis.

High-fructose diets have induced fatty liver in rats[53] and ducks[54], such diets have also caused increases in hepatic lipid peroxidation[55] and activation of inflammatory pathways in the liver of rats[21].

Fructose is lipogenic and stimulates TG synthesis[31]. Splanchnic perfusion studies demonstrate that fructose produces higher rates of TG secretion from the liver than equimolar amounts of glucose[56]. The long-term administration of fructose to rats results in hepatic macro- and micro vesicular steatosis with a 198% increase in hepatic TGs and an 89% increase in hepatic cholesterol concentration[57]. Furthermore, the administration of a diet with 25% of the total energy as sucrose (which contains 50% fructose) resulted in a rise in hepatic aminotransferases (ALT and AST) levels within 18 d[58,59]. Indeed, total fructose intake averages approximately 12% of the total energy intake and may increase to 15% in some subgroups in the US population[22].

Animals maintained on a chronic high fructose diet develop elevated non-esterified fatty acids (NEFAs) and hyperinsulinemia at the expense of glycemic control[39]. This is not surprising, as fructose-induced metabolic dyslipidemia is usually accompanied by whole body insulin resistance[60] and reduced hepatic insulin sensitivity[61].

A potential mechanism by which fructose may cause liver injury is shown in Figure 5. The metabolism of fructose is distinct from glucose. Before converging with the glycolytic pathway, initial fructose metabolism involves phosphorylation of fructose to fructose-1-phosphate by fructokinase (ketohexokinase, KHK) using the substrate ATP. Unlike glucokinase, the phosphorylation of fructose by fructokinase is specific for fructose and not rate limited. The high activity of fructokinase in phosphorylating fructose to fructose-1-phosphate in the liver, can result in hepatic ATP depletion[22]. Indeed, fructose has been shown to cause ATP depletion in humans[62,63], and recovery from fructose-induced ATP depletion was found to be delayed in subjects with NALFD in studies that used phosphorus-1 magnetic resonance spectroscopy to assess hepatic metabolism[63,64]. In some respects, fructose-induced ATP depletion resembles hepatic ischemia[65]. In rats, fructose administration increases hepatic lipid peroxidation and activation of inflammatory pathways[21,55]. Cirillo *et al*[66] found that incubation of endothelial cells or renal tubular cells with postprandial concentrations of fructose reduces intracellular ATP and activates proinflammatory and prooxidative responses. Therefore, high fructose consumption may contribute to NAFLD pathogenesis because fructose-induced ATP depletion promotes hepatic necroinflammation. Moreover, fructose promotes insulin resistance, lipid peroxidation, dyslipidemia, increased arterial blood pressure, increased AGEs, and increased hepatic inflammation[66].

Ouyang *et al*[67] found that subjects with NAFLD have a significantly greater intake of sweetened beverages by history, representing a 2-fold greater intake than the mean intake in both controls and in population-based studies. Their second finding was that the key initiating enzyme in fructose metabolism, KHK (ketohexokinase), was also increased 2-fold in the liver biopsies of these patients compared to controls[67]. The increase in KHK levels is consistent with the known effect of fructose to upregulate KHK in the liver of rats[68,69].

Patients on a high fructose or sucrose diet show a greater uric acid response to a bolus of fructose[70,71] consistent with the upregulation of KHK activity. Finally, uric acid levels can predict the development of NAFLD[72]. There is also increasing evidence that the rise in uric acid may also have a potential role in causing features of the metabolic syndrome[73], in part by the ability of uric acid to deplete endothelial nitric oxide levels[74] and by activating adipocytes[68]. What does fructose become in our liver? Fructose becomes free fatty acids (the building blocks of all lipids), becomes VLDL lipoproteins and TGs (the nasty lipids most associated with cardio- vascular disease), and becomes uric acid (oxidative stress, vascular inflammation, Figure 5).

FRUCTOSE AND METABOLIC SYNDROME

Reaven noted that several risk factors (e.g. dyslipidemia, hypertension and hyperglycemia) are commonly clustered together[75]. This clustering he called Syndrome X, and he recognized it as a multiplex risk factor for cardiovascular disease (CVD). Other researchers use the term metabolic syndrome for this clustering of metabolic risk factors. ATP Ⅲ used this alternative term[76]. Beyond CVD and type 2 diabetes, individuals with metabolic syndrome are susceptible to other conditions, notably polycystic ovary syndrome, fatty liver, cholesterol gallstones, asthma, sleep disturbances, some forms of cancer, and is associated with a proinflammatory/prothrombotic state that include elevated levels of C-reactive protein, endothelial dysfunction, hyperfibrinogenemia, increased platelet aggregation, increased levels of plasminogen activator, elevated uric acid levels, microalbuminuria, and a shift toward small, dense particles of low-density lipoprotein[77].

The major characteristics of metabolic syndrome include insulin resistance, abdominal obesity, elevated blood pressure, and lipid abnormalities (i.e. elevated levels of TGs and low levels of HDL cholesterol).

The role of fructose in insulin resistance, hyperglycemia, and obesity that constitute important elements of the metabolic syndrome were discussed above[77].

Visceral adipose tissue and dyslipidemia induced by fructose/sucrose consumption play a major role in the development and progression of metabolic syndrome. The main role of adipose tissue is to take up excess fatty acids provided by the diet and to store them in the form

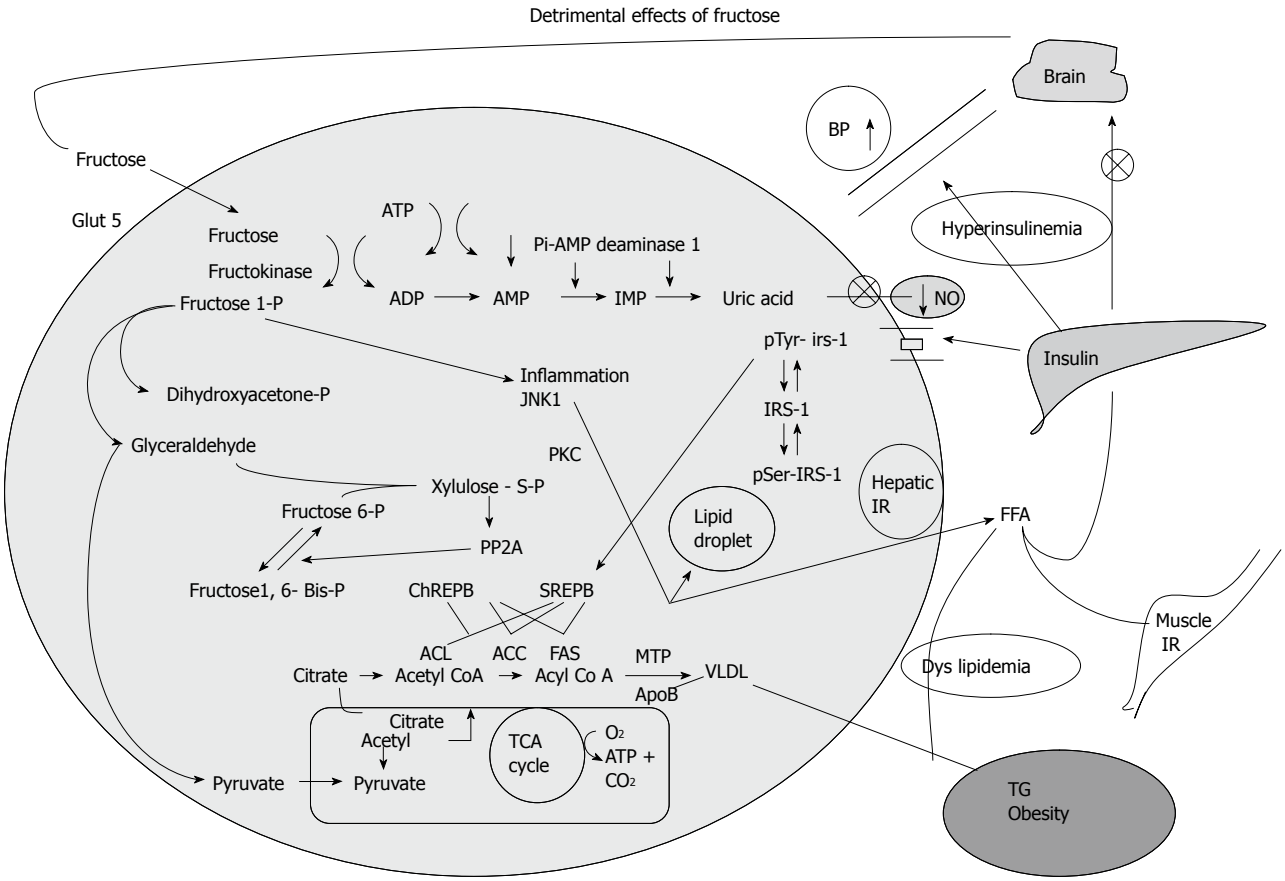


Figure 5 Mechanisms of detrimental effects of fructose.

of TGs to be used as an energy supply for the body in times of starvation, however, adipose tissue has a limited capacity to store fat. This maximum capacity may be reached in states of obesity, resulting in an impaired ability of adipose tissue to acquire dietary fatty acids and, therefore, increased levels of fatty acids are found in the circulation[77].

Signaling abnormalities in adipocytes can also trigger lipolysis of TG stores and the efflux of fatty acids into the bloodstream, augmenting the problem. The presence of high levels of NEFAs in the bloodstream is proposed to function as a key mechanistic link between obesity and insulin resistance, type 2 diabetes, and metabolic dyslipidemia. Eventually, these NEFAs may be taken up ectopically by non-adipose tissues such as the liver and skeletal muscle, where they may be stored as TG or diacylglycerol and interfere with metabolic pathways such as the response to insulin, contributing to insulin resistance and the metabolic syndrome[78].

Differences exist in the metabolic properties of the various sites of adipose tissue. Visceral or abdominal fat stores are believed to pose a greater risk for the development of insulin resistance and the metabolic syndrome than subcutaneous fat stores. Reasons for this include reduced responsiveness of visceral fat to the anti-lipolytic effects of insulin (due to lower expression and activity of hormone sensitive lipase, reduced tyrosine phosphorylation of the insulin receptor, decreased IRS-1 expression, and increased PTP-1B activity); greater responsiveness of visceral fat to the lipolysis-inducing effects of catecholamines; and decreased uptake and acylation of fatty acids compared with subcutaneous fat, all of which result in amplification of NEFA levels in the blood[79]. Visceral fat is also located conveniently for these NEFAs to enter the portal circulation for direct delivery to the liver, where they pose a risk to hepatic insulin responsiveness.

Fructose consumption can induce perturbations in cell signaling and inflammatory cascades in insulin-sensitive tissues[25]. The contribution of fructose/sucrose in dyslipidemia was discussed above. Consuming such large amounts of fructose/sucrose can lead to the development of a complete metabolic syndrome by increasing plasma TGs and altering hepatic glucose homeostasis, gaining weight, and decreasing insulin sensitivity.

CONCLUSION

The use of sweeteners has increased considerably worldwide and soft drink beverages seem to be a major contributor for obesity, diabetes mellitus, hyperlipidemia, insulin resistance, hypertension, metabolic syndrome, and cardiovascular disease. In this review we sought to focus attention on the impact of soft drinks on the accumulation of fat in the liver. This has significant clinical implications, as

the presence of NAFLD correlates strongly with diabetes, cardiovascular disease and diffuse atherosclerosis.

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EDITORIAL

Prediction of disease course in inflammatory bowel diseases

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Abstract

Clinical presentation at diagnosis and disease course of both Crohn's disease (CD) and ulcerative colitis are heterogeneous and variable over time. Since most patients have a relapsing course and most CD patients develop complications (e.g. stricture and/or perforation), much emphasis has been placed in the recent years on the determination of important predictive factors. The identification of these factors may eventually lead to a more personalized, tailored therapy. In this TOPIC HIGHLIGHT series, we provide an update on the available literature regarding important clinical, endoscopic, fecal, serological/routine laboratory and genetic factors. Our aim is to assist clinicians in the everyday practical decision-making when choosing the treatment strategy for their patients suffering from inflammatory bowel diseases.

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Key words: Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Disease course; Predictive markers; Clinical; Serology; Genetics

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In this TOPIC HIGHLIGHT series, we provide an update on the available literature regarding important clinical, endoscopic, fecal, serological/routine laboratory and genetic factors (Table 1) that may be used for the prediction of the disease course in inflammatory bowel diseases (IBD)[1]. Our aim is to assist clinicians in the everyday practical decision-making when choosing the treatment strategy for their patients suffering from IBD.

We focus first on clinical and endoscopic variables by Louis *et al*[2] and Allez *et al*[3] that were found to be important medium and long-term markers in the prediction of complicated disease course in IBD. The phenotypic classification of Crohn's disease (CD) plays an important role in determining the treatment, and may assist in predicting the likely clinical course of disease. Using the Vienna classification system, it has been shown in clinic-based cohorts that there can be a significant change in disease behavior over time, whereas disease location remains relatively stable[4,5]. In addition, factors, e.g. young age at onset or pediatric presentation, early steroid use, ileal disease, presence of perianal disease, mucosal healing or smoking were identified as an important risk factor for developing disabling disease or major abdominal surgery[6-10]. Fewer factors are available in ulcerative colitis. One of the important factors is disease extent, identified by previous studies and also a recent 10-year population-based inception cohort study[7].

A second group of markers that will be discussed in details are biomarkers. Biomarkers can be divided into long-term markers (e.g. genetics and serology) influencing the clinical phenotype and short-term markers that may be used for the prediction of flares. Genetic (e.g. nucleotide oligomerization domain, NOD2)/caspase activation recruitment domain (CARD15) and serology markers, discussed by Vermeire *et al*[8] and Dubinsky *et al*[9], may be important in the prediction of long term evolution of disease phenotype and need for surgery in CD or they might also be used to predict the efficacy and/or side effects of medical therapy. However, various geographical differences may exist at least for genetic

Table 1 Predictors for long and short term disease course in inflammatory bowel diseases

Markers
Disabling/complicated disease in CD
Young age at onset (pediatric or < 40 yr)
Small bowel disease
Stricturing
Perianal disease
Weight loss > 5 kg
Steroid need for the first flare at diagnosis
Early immunosupression and/or biological therapy (protective?)
NOD2/CARD15
rs1363670 near IL12B
ASCA, glycans
Surgery in CD/colectomy in UC
Young age at onset (pediatric or < 40 yr)
Small bowel disease
Stricturing or penetratingat diagnosis
Early immunosupression and/or biological therapy (protective?)
Deep ulcers at endoscopy
ASCA, glycans
Extensive disease in UC
Smoking (protective) in UC
Clinical relapse
CRP, ESR
Calprotectin
Complete or partial mucosal healing (protective)

NOD2/CARD15: Nucleotide oligomerization domain2/caspase activation recruitment domain15; ASCA: Anti-*Saccharomyces cerevisiae* antibodies; CRP: C reactive protein; ESR: Erythrocyte sedimentation rate; UC: Ulcerative colitis; CD: Crohn's disease.

markers. Finally, we focus on the predictive power of serum, fecal and mucosal markers, which were used by Scaldaferri *et al*[10] to identify relapses and/or disease progression.

The search for clinical, laboratory, and molecular markers to define and predict disease outcome in common diseases has moved rapidly forward with the help of modern technologies and vigorous collection of clinical data. However, many questions remain unanswered. We believe that this issue will be of strong interest not only for IBD specialists, but also for gastroenterologists, since our ultimate goal is to identify individual patient profiles, including clinical, laboratory and molecular markers, which will hopefully allow us to choose the most appropriate management in terms of therapy and intensity of follow-up.

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TOPIC HIGHLIGHT

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Is the disease course predictable in inflammatory bowel diseases?

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Abstract

During the course of the disease, most patients with Crohn's disease (CD) may eventually develop a stricturing or a perforating complication, and a significant number of patients with both CD and ulcerative colitis will undergo surgery. In recent years, research has focused on the determination of factors important in the prediction of disease course in inflammatory bowel diseases to improve stratification of patients, identify individual patient profiles, including clinical, laboratory and molecular markers, which hopefully will allow physicians to choose the most appropriate management in terms of therapy and intensity of follow-up. This review summarizes the available evidence on clinical, endoscopic variables and biomarkers in the prediction of short and long-term outcome in patients with inflammatory bowel diseases.

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Key words: Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Disease course; Predictive markers; Clinical; Serology; Genetics

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INTRODUCTION

Inflammatory bowel disease (IBD) is a multifactorial disease with probable genetic heterogeneity[1]. In addition, several environmental risk factors (e.g. diet, smoking, measles or appendectomy) may contribute to its pathogenesis. During the past decades, the incidence pattern of various forms of IBD has changed significantly[2], showing some common but also quite distinct characteristics for the different variations of the disorder.

The phenotypic classification of Crohn's disease (CD) plays an important role in determining the treatment, and may assist in predicting the likely clinical course of disease[3]. In 2005, the Montreal revision of the Vienna classification system was introduced[4]. Using the Vienna classification system, it has been shown in clinic-based cohorts that there can be a significant change in disease behavior over time, whereas disease location remains relatively stable[3,5]. Since the early introduction of immunomodulators and/or biologicals might be justified in patients at risk for disease progression, so it is important to identify these patients as soon as possible. Much emphasis has been placed in recent years on the determination of important predictive factors. In this review, the authors try to highlight some of the clinical, endoscopic variables and biomarkers that are important in the prediction of disease course in IBD (Table 1).

CLINICAL, ENVIRONMENTAL, AND ENDOSCOPIC FACTORS

Clinical and endoscopic variables are important medium

Table 1 Possible use of predictors for long and short term disease course in inflammatory bowel diseases

	Marker name	Clinical outcome
Clinical markers	Young age at onset (pediatric or < 40 yr)	Disabling disease
	Small bowel disease	
	Stricturing	
	Perianal disease weight loss > 5 kg	
	Steroid need for the first flare at diagnosis	
	Early immunosupression and/or biological therapy (protective?)	
	Young age at onset (pediatric or < 40 yr)	Surgery
	Small bowel disease	
	Stricturing or penetrating at diagnosis	
	Early immunosupression and/or biological therapy (protective?)	
	Extensive disease	Colectomy in UC
	Younger age at onset (< 50 yr)	
	Smoking (protective)	
Genetic markers	NOD2/CARD15	Disease location/behavior/surgery in CD
	rs1363670 near IL12B	Disease behavior
	TPMT	Azathioprine toxicity
Serology markers	ASCA, glycans	Complicated disease, surgeries
Biomarkers	CRP	Clinical flares
	ESR	
	Calprotectin	
Endoscopy markers	Complete or partial mucosal healing (protective)	Clinical flares and surgery

NOD2/CARD15: Nucleotide oligomerization domain2/caspase activation recruitment domain15; TPMT: Thiopurine S-methyltransferase; ASCA: Anti-*Saccharomyces cerevisiae* antibodies; CRP: C reactive protein; ESR: Erythrocyte sedimentation rate; CD: Crohn's disease; UC: Ulcerative colitis.

and long-term markers in the prediction of complicated disease course in IBD. A young age at onset or pediatric presentation is an important risk factor for developing complicated disease behavior, and even disease extension in a large proportion of patients, as reported in a recent French population based-cohort study[6,7].

In CD, an initial need for steroid use (OR: 3.1, 95% CI: 2.2-4.4), an age below 40 years (OR: 2.1, 95% CI: 1.3-3.6), and the presence of perianal disease (OR: 1.8, 95% CI: 1.2-2.8) were associated with the development of disabling disease in the study by Beaugerie *et al*[8]. The positive predictive value of disabling disease in patients with 2 and 3 predictive factors of disabling disease was 0.91 and 0.93, respectively. More recently, authors from New Zealand[3] have shown that > 70% percent of CD patients had inflammatory disease at diagnosis, with the proportion of patients with complicated disease increasing over time. Progression towards complicated disease was more rapid in those with small bowel than colonic disease location, ($P < 0.001$), and perianal disease was a significant predictor of change in CD behavior [hazard ratio (HR): 1.62, $P < 0.001$]. Similarly, perianal lesions, the need for steroids to treat the first flare and ileocolonic location, but not age below 40 years were confirmed as predictive markers for developing disabling disease (according to the predefined criteria) at 5 years[9]. In the same study, stricturing behaviour (HR: 2.11, 95% CI: 1.39-3.20) and weight loss (> 5 kg) (HR: 1.67, 95% CI: 1.14-2.45) at diagnosis were independently associated with the time to development of severe disease. In addition, small bowel location and stricturing disease were predictors for surgery in a long term follow-up study[10]. Finally, terminal ileal location ($P < 0.001$), stricturing ($P = 0.004$), penetrating behavior ($P < 0.001$), and age younger than 40 years ($P = 0.03$) at diagnosis were independent risk factors for subsequent surgery in a prospective 10-year follow-up study by the IBSEN group[11].

An important environmental factor that may possibly be important in determining changes in disease behavior in CD is smoking. In CD, smoking was reported to be associated with disease location: most, but not all, studies report a higher prevalence of ileal disease and a lower prevalence of colonic involvement in smokers[12,13]. A recent review[13] and previous data have demonstrated that smoking, when measured up to the time-point of disease behavior classification, was associated more frequently with complicated disease, penetrating intestinal complications[12,14,15], and greater likelihood to progress to complicated disease, as defined by the development of strictures or fistulae[13], and a higher relapse rate[16]. In addition, the risk of surgery as well as the risk for further resections during disease course was also noted to be higher in smokers in some studies[12,17] and in a recent meta-analysis[18]. The need for steroids and immunosuppressants was found to be higher in smokers compared to non-smokers[19]. In a recent paper by Aldhous *et al*[20] using the Montreal classification, the harmful effect of smoking was only partially confirmed. Although current smoking was associated with less colonic disease, smoking habits at diagnosis were not associated with time to development of stricturing disease, internal penetrating disease, perianal penetrating disease, or time to first surgery.

In one study by Cosnes *et al*[21], immunosuppressive therapy was found to neutralize the effect of smoking on the need for surgery. The importance of an early aggressive medical strategy in determining long term disease phenotype is further supported by a recent Hungarian study[22,23] where the authors have reported that early aza-

thioprine (AZA)/biological therapy reduced the risk for disease behavior change and delayed the time to the first operation in CD in both smokers and non-smokers. The most convincing data to support a benefit from early use of AZA however, comes from the pediatric literature[24], where in a randomized controlled trial in 55 children, the early use of 6-mercaptopurine (6-MP) was associated with a significantly lower relapse rate (only 9%) compared with 47% in controls ($P = 0.007$). Moreover, the duration of steroid use was shorter ($P < 0.001$) and the cumulative steroid dose was lower at 6, 12, and 18 mo ($P < 0.01$).

In addition, in a recent withdrawal study by the Groupe D'Etude Thérapeutique Des Affections Inflammatoires Du Tube Digestif (GETAID) group[25], authors have provided evidence for a benefit of long term AZA therapy beyond 5 years in patients with prolonged clinical remission. Finally, in a recent controlled randomized prospective trial[26], AZA administered for 12 mo together with metronidazole for 3 mo was more effective in preventing endoscopic postoperative recurrence assessed at 12 mo, compared to metronidazole alone in patients previously only minimally exposed to AZA.

Fewer factors are available in ulcerative colitis (UC). One of the important factors is disease extent, identified by previous studies and also a recent 10 years population-based inception cohort study[27]. In the same study, an age at the time of diagnosis > 50 years was associated with reduced hazard ratio (0.28) for subsequent colectomy. In addition, one-fifth (69/288) of patients with proctitis or left-sided colitis had progressed to extensive colitis. In contrast, pediatric UC was more aggressive. Disease course was characterized by disease extension in 49% of patients during in a 6.5 years population-based cohort study[7]. A delay in diagnosis of more than 6 mo and a family history of IBD were associated with an increased risk of disease extension, with odds ratios of 5.0 (1.2-21.5) and 11.8 (1.3-111.3), respectively. The cumulative rate of colectomy was 8% at 1 year, 15% at 3 years, and 20% at 5 years. Of note, however, surgical rates have shown a wide geographical variation with significantly lower rates reported from the Mediterranean region and Eastern Europe in earlier studies[22,28].

Furthermore, in contrast to CD, smokers with adult UC have been reported to run a more benign disease course compared to non-smokers[29]. Flare-ups, hospitalization rates, the need for oral steroids, and colectomy rates, were reported to be lower, while age at onset was older in smokers compared to non-smokers, though not in all studies. Relapse rates were lower in patients who began smoking after the diagnosis of UC was made. In concordance, in a recent Europe-wide population-based cohort[30], the relapse rate was lower (HR: 0.8, 95% CI: 0.6-0.9) in smokers compared to non-smokers, while being higher in women. In addition, in a meta-analysis of several large series with a total of 1489 UC patients, the risk for colectomy was lower (OR: 0.57, 95% CI: 0.38-0.85) in current smokers compared to non-smokers[31].

Finally, it is still unclear whether mucosal healing prevents the development of complications, although its importance was investigated in several studies using heterogeneous definitions. A well-known example is the study by Allez *et al*[32] where severe endoscopic activity was associated with an increased risk for colectomy in CD after 1, 3 and 8 years of follow-up. This was only partially confirmed in a more recent population-based follow-up study by the IBSEN group, where mucosal healing at 1 year in UC was associated with a lower subsequent colectomy risk, but mucosal healing in CD was not a significant predictor for resective surgery at 5 years, but the risk of future steroid use was decreased[33]. Similarly, in a very recent follow-up report the beneficial effect of early complete mucosal healing was confirmed in early CD. Complete mucosal healing in patients with early-stage CD was associated with significantly higher steroid-free remission rates 4 years after the start of the therapy[34]. Similarly, mucosal healing at 6 mo following the start of scheduled infliximab maintenance therapy was associated with lower need for major abdominal surgery during follow-up in a study from the Leuven group[35].

BIOMARKERS

Biomarkers can be divided into long-term markers (e.g. genetics and serology) influencing the clinical phenotype and short-term markers that may be used for the prediction of flares.

In 2001, the identification of nucleotide oligomerization domain2 (NOD2)/caspase activation recruitment domain15 (CARD15) on chromosome 16 in the IBD1 region, as a candidate gene for CD[36,37], has stimulated further research focusing on novel genetic determinants of susceptibility and phenotype in IBD. The presence of a NOD2/CARD15 mutation increases the risk for CD by 1.4-4.3-fold in heterozygous patients and 17.6-44.0-fold in homozygous and compound heterozygous patients. Of importance, reports exist of homozygous individuals who are disease-free. However, various geographical differences were noted. A much lower prevalence of these mutations was reported in, for example African American, Chinese and Japanese[38] populations. NOD2/CARD15 is a good example for case-control phenotype-genotype correlations, since in earlier studies the three common NOD2/CARD15 mutations were associated with ileal disease and fibrostenosing behavior. On the contrary, in colonic and fistulizing disease, they were relatively less frequent[39,40]. There are many controversial findings, such as the association between the OCTN TC risk haplotype and penetrating disease (OR: 1.474, 95% CI: 1.028-2.114, $P = 0.035$) in one study[41] or non-fistulizing non-fibrostenotic disease (OR: 1.57), colonic disease (OR: 1.31), an earlier age at the onset of disease, and reduced need for surgery (OR: 1.38) in another[42], or the association between the presence of DLG5 R30Q variant and steroid refractory disease that could not be confirmed yet[43]. Finally, in a recent study by Weersma *et al*[44] the same type of statistical analysis was performed in a little more complex genetic dataset by using both novel markers (e.g. ATG16L1,

IL23R) and earlier (NOD2, IBD5 and DLG5) genetic markers. In this study, patients with CD with a more severe disease course, surgeries or an age of onset below 40 years had more risk alleles compared to non-stricturing, nonpenetrating behaviour (P = 0.0008), no operations (P = 0.02) or age at onset greater then 40 years (P = 0.028). However, the association became insignificant after a longer disease duration, confirming that many other factors (e.g. clinical variables, disease phenotype at diagnosis, medical therapy) contribute to the long term evolution of disease phenotype.

Although in some of these studies the authors corrected their findings for disease duration using logistic regression, these are all static correlations between phenotype and genotype. In 2009, we finally observed new examples of genotype-phenotype association scans, where the authors have performed a complete follow-up of the patients and so the association between time-dependent clinical outcomes and genotype could be more preciously evaluated in multivariate analysis using Cox regression. Moreover, the authors realized that clinical parameters should also be included in the complex testing. A fine example of how this should be performed was recently published by the Leuven group[45]. Of course, the presentation of the results is now much more complex. For example, homozygosity for the rs1363670 G-allele in a gene encoding a hypothetical protein near the *IL12B* gene was independently associated with stricturing disease behavior and with shorter time to strictures, especially in patients with ileal involvement, or alternatively male patients carrying at least one rs12704036 T-allele in a gene desert had the shortest time to non-perianal fistula. This landmark study with its complex analysis is, however, bringing genetics closer to being used in routine clinical practice.

Genetic markers may also be used to predict the efficacy and/or side effects of medical therapy, that is pharmacogenetics. This use is also expected to be complex and include certain clinical predictors. The importance of using conventional genetic testing might be highlighted by the well-known thiopurine S-methyltransferase (TPMT) genotype/activity testing. TPMT limits the production of 6-TGNs by converting 6-MP to 6-thioruric acid and 6-methylmercaptopurine[46], which may lead to myelotoxicity. Population studies have shown that the distribution of TPMT activity is trimodal: 0.3%-0.5% of the population has low to absent activity (TPMTL/TPMTL), around 10% have intermediate activity (TPMTL/TPMTH), and approximately 90% inherit normal to high enzyme activity (TPMTH/TPMTH)[46]. In this regard, a correlation between the TPMT genotype and enzyme activity has been proven. Approximately 5% of the white population carries one or more variant TPMT alleles, with more than ten variant alleles reported[47]. The functional consequences of alleles *2, *2A, *3B, and *3C, accounting together for more than 90% of mutant alleles, have been extensively characterized. Nevertheless, it is clear that there are many other causes of myelotoxicity. This was accurately demonstrated by Colombel *et al*[48], who found that only 27% of patients had a documented low TPMT activity. Other confounding genetic and environmental factors include, for instance, the patient's age, renal function, AZA formulation, coadministration of mesalazine (a reversible TPMT inhibitor) and allopurinol (XO inhibitor). Thus, the determination of TPMT activity is not an exclusive test to rely on. It may only be helpful in identifying a certain group of high risk patients but as the negative predictive value is rather low, it is not beneficial in ruling out possible side effects. Also, as the prevalence of double carriage of variant TPMT alleles is as low as 1/300, continuous monitoring of red blood cell counts remains mandatory in clinical practice. A different approach was recently reported by the Leuven group[49]. The authors analysed gene expression signatures in the mucosa of patients with UC and were able to identify predictive panels of genes for (non-)response to infliximab. Further studies of the pathways involved should allow a better understanding of the mechanisms of resistance to infliximab therapy in UC.

Similarly, serological markers represent medium to long term markers of disease phenotype and progression. The phenotype and disease course of CD are associated with the presence and extent of the serologic response targeted against various microbial antigens. In patients with conventional anti-*Saccharomyces cerevisiae* antibodies (ASCA$^+$, IgG and/or IgA) or atypical perinuclear antineutrophil cytoplasmic antibodies (P-ANCA) phenotype, small bowel involvement (with or without colonic disease) is more typical than the pure colonic disease (60%-80% *vs* 30%-45%)[50,51]. ASCA positivity predicts a more aggressive disease course with a higher rate of complications. ASCAs have been associated with stricturing and penetrating disease and a higher risk of small bowel resection[51,52]. Some studies also suggest that ASCA positivity is associated with an earlier onset of disease. The presence of anti-OmpC in adult CD patients is associated with an increased prevalence of the penetrating form only[53], while in children, both the penetrating and stenosing forms[54] are more frequent. Moreover, antibody positivity may lead to a more aggressive course of disease and a higher risk for surgical interventions. Like ASCA and anti-OmpC, anti-I2 also appears to be associated with an increased risk for complications in adult CD patients. It is an independent risk factor for the development of the stenosing form and the need for surgical interventions[54]. Recent research has shown that the anti-CBir1 antibody is associated with ileal involvement in adult CD patients independently from other serological markers, and it predisposes for the development of both stenosing and penetrating forms[55]. Interestingly anti-Cbir1 was more frequent in young children with CD (age: 0-7 years)[56]. In a very recent study, an association was also reported between the presence of pancreatic autoantibodies and perforating perianal disease, arthritis, ocular, and cutaneous manifestations[57]. In addition to qualitative correlations, quantitative correlations with serological responses are also present. The number of antibodies produced against microbial anti-

gens in CD also showed a positive correlation with the severity of the disease course[51].

Similarly, the presence and magnitude of the more newly developed antiglycan antibodies (including the most recent anti-laminarin and anti-chitin markers) in CD was associated with a more aggressive disease phenotype in CD, as well as a younger age at onset, ileal involvement, stricturing/penetrating disease behavior, and risk for surgery[58-60]. In the latter study, gASCA IgG and ASCA IgG were comparable for the diagnosis and determination of disease phenotype in CD; however, each test recognized an additional patient population that was missed by the other, indicating a partially complementary role. The differentiation between colon only CD and UC was also better[58,61].

In prospective pediatric cohorts Dubinsky *et al*[54], found that the presence and magnitude of immune responses to microbial antigens (ASCA, anti-OmpC, anti-I2 and anti-CBir1) are significantly associated with more aggressive disease phenotype. The risk of developing penetrating and/or stricturing CD was increased in those individuals with immune response against all four microbial antigens compared to seronegative cases. Moreover, they demonstrated that the time to develop a disease complication during the 18 mo of follow-up period is significantly faster in those children who have a serologic response against at least one antigen, recently confirmed by the same group in a much larger IBD population[62].

In addition, the occurrence of atypical P-ANCA in UC is associated with a characteristic clinical appearance and represents a distinct subgroup, which is often characterized by specific human leucocyte antigen markers. These patients have a higher probability of developing a severe left-sided UC that is more resistant to treatment than the usual case. The disease has a more aggressive course requiring surgery earlier in the course of the disease[63]. The presence of atypical P-ANCA identifies a subgroup of CD patients characterized by "UC-like" colitis; the inflammation usually involves the left side of the colon and the response to therapy is generally good. The atypical P-ANCA in CD patients is associated with a later age of onset and a relatively smaller incidence of complications, such as stricture and/or perforation[64].

Of note however, one should be aware of the fact that the serology profile is not a stable phenotype; seroreactivity may change during the course of follow-up. This is at least suggested by studies done in patients with celiac disease where seropositivity was lost several months after the introduction of a gluten free diet[65].

IBD follows an alternating disease course and both CD and UC are characterized by periods of remission and relapse. However, disease flare-ups occur in a random way and are often unpredictable. Based on the half-life and changes over time serum laboratory and fecal markers may be best used for the prediction of short or medium term relapses. Erythrocyte sedimentation rate (ESR) and C reactive protein (CRP) are traditional non-specific markers of inflammation. In addition, a number of neutrophil-derived proteins present in stool samples have been studied, including fecal lactoferrin, lysozyme, elastase, myeloperoxidase, and calprotectin.

ESR is defined as the rate at which erythrocytes migrate through the plasma. Inevitably, ESR will depend on the plasma concentration and on the number and size of the erythrocytes. Conditions such as anemia, polycythemia, and thalassemia affect ESR. Compared with CRP, ESR will peak much less rapidly and may also take several days to decrease, even if the patient's clinical condition or the inflammation is ameliorated. Although CRP is non-specifically upregulated in most inflammatory diseases, including IBD, there is remarkable heterogeneity in the CRP response between CD and UC. Whereas CD is associated with a strong CRP response, UC has only a modest or no CRP response in both adult and children IBD populations[66]. This is an important fact to keep in mind when using CRP in clinical practice. There is no good explanation for this heterogeneity.

In general, patients with severe disease more often have abnormal inflammatory markers, compared with patients without or with only a low-grade inflammation. This has been shown in one of the early studies by Fagan *et al*[67], both CRP and ESR correlated well with disease activity, yet the correlation was better for CRP. In addition, the correlation of laboratory markers with disease activity has been shown to be much stronger for CD than for UC. However, a wide range of CRP values was observed and no clear cut-off values exist between mild to moderate (10-50 mg/L), moderate to severe (50-80 mg/L), and severe disease (> 80 mg/L). Therefore, the comparison of individual CRP values with previous values in any given patient is of great clinical importance. In addition, CRP showed acceptable correlation with endoscopic and histological activity in CD[68]. For UC, again, this correlation was weaker. In contrast, more recent data from Australia suggested that a subgroup of patients with active ileal disease, low body mass index, and previous ileocecal resection was characterized by low CRP[69].

In CD, a number of studies have investigated a panel of laboratory markers in predicting clinical relapse. Later, Boirivant *et al*[70] prospectively followed 101 outpatients with CD. Half of the patients had an elevated CRP value and this correlated well with clinical activity. Approximately one-third of CD patients presented with active disease despite normal CRP and one-third had high CRP levels but clinically inactive disease. The likelihood of relapse after 2 years was higher in the patients with an increased CRP compared with those who had a normal CRP value. More recently, the GETAID group[71] proposed a simple biological score for predicting short-term relapse in CD. Multivariate analysis selected two markers predictive of relapse: CRP > 20 mg/L and an ESR > 15 mm/h. The relative risk of short-term relapse for patients with a positive score compared to those with a negative score was 8.0 (95% CI: 2.8-22.9). The score's sensitivity and specificity was 89% and 43%, respectively, with a negative predictive value of 97%, suggesting that normal CRP and ESR could almost certainly rule out a relapse in the next 6 wk. Much less data exist on the value of laboratory mark-

ers in assessing disease course and outcome in UC. The well known, prospective study from Oxford evaluated 49 severe UC patients treated with hydrocortisone and/or cyclosporine (n = 49). On day 3, a daily stool frequency of > 8 or 3-8 with increased CRP (> 45 mg/L) predicted with 85% certainty the need for colectomy[72]. Only one relatively small study investigated the role of hs-CRP in IBD[73]. Ninety CD, 70 UC patients, and 160 controls were investigated. The coefficient of correlation between hs-CRP and the disease activity score was weak but similar in both UC (0.26) and CD (0.36).

Finally, a recent population based study by the IBSEN group has demonstrated[74] that CRP at diagnosis or during follow-up was able to predict medium term outcome as measured by the need for colectomy or resective surgery. In patients with UC with extensive colitis, CRP levels above 23 mg/L at diagnosis predicted an increased risk of surgery (OR: 4.8, 95% CI: 1.5-15.1) during the first 5 years of follow-up. In patients with UC, CRP levels above 10 mg/L after one year predicted an increased risk of surgery during the subsequent 4 years (OR 3.0, 95% CI: 1.1-7.8). A significant association between CRP levels at diagnosis and risk of surgery was also found in patients with CD with terminal ileitis (L1), where the risk increased when CRP levels were above 53 mg/L in this subgroup (OR 6.0, 95% CI: 1.1-31.9).

A change in CRP following therapy serves as a good parameter in assessing the effectiveness of the drug on the underlying inflammation. A decrease in CRP, in response to therapy, is objective evidence that the drug has a beneficial effect on intestinal inflammation, even in patients with small changes in their symptoms. On the other hand, persistently raised CRP indicates therapeutic failure in controlling mucosal inflammation. This was clearly demonstrated by the different response rates to anti-TNF antibodies in patients with CD. In some studies, a high baseline (5-10 mg/L) CRP value before the start of therapy was associated with a higher response compared to patients with lower CRP[75,76]. These findings raise the question of whether CRP should be included in patient selection for future clinical trials, at least for selected drugs. Furthermore, high CRP (> 20 mg/L) was found to be an independent predictor for relapse after AZA withdrawal in patients on AZA therapy for longer than 42 and 63 mo[25,77].

Fecal calprotectin was also shown to predict relapse in CD. Calprotectin, a 36 kDa calcium- and zinc-binding protein, is probably the most promising marker for various reasons. In contrast with other neutrophil markers, calprotectin represents 60% of cytosolic proteins in granulocytes. The presence of calprotectin in feces can therefore be seen as directly proportional to neutrophil migration to the gastrointestinal tract. Fecal calprotectin is a very stable marker (stable for more than 1 wk at room temperature) and is resistant to degradation, making it an attractive option. Other neutrophil protein markers, include fecal lactoferrin, polymorphonuclear-elastase, and S100 A12. They are also more specific to intestinal rather than systemic inflammation, but their clinical role has been less extensively evaluated in IBD.

In the study by Tibble *et al*[78], calprotectin levels of 50 μg/g or more predicted a 13-fold increased risk for relapse. In another study[79], 38 CD and 41 UC patients were investigated. All patients were in remission for a mean duration of 5 mo. A baseline level of calprotectin of 150 μg/g or more was predictive for a relapse in the next year. Although sensitivity was high for both CD (87%) and UC (89%), specificity was much lower in the case of CD (43%) compared to UC (82%). Similarly, in a recent study, fecal calprotectin and lactoferrin were able to differentiate organic colorectal disease and were associated with active disease at endoscopy, both in UC (78% and 75%, respectively) and in CD (87% and 82%, respectively)[80]; however, specificity was low overall. It is thus difficult to define cut-off values based on these studies. In addition to serum markers, fecal calprotectin also correlates well with endoscopic and histological activity in patients with UC and CD, and elevated calprotectin levels normalize once the inflammation has resolved in both adults and children[81,82].

Lastly, calprotectin, at a cut-off value of 200 mg/L, at 3 mo post-surgery, was useful in predicting endoscopic post-surgical recurrence in asymptomatic patients with a sensitivity of 63% and a specificity of 75%[83].

CONCLUSION

In conclusion, the search for clinical, laboratory, and molecular markers to define and predict disease outcome in common diseases has moved rapidly forward with the help of modern technologies and vigorous collection of clinical data. However, many questions remain unanswered. Nevertheless, research should be encouraged because, as physicians treating patients with IBD, our ultimate goal is to have at our disposal a complex individual patient profile, including clinical, laboratory and molecular markers around the time of diagnosis which will hopefully allow us to choose the most appropriate management in terms of therapy, intensity of follow-up and frequency of various investigations.

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TOPIC HIGHLIGHT

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Do clinical factors help to predict disease course in inflammatory bowel disease?

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Abstract

While therapeutic strategies able to change the natural history of the disease are developing, it is of major importance to have available predictive factors for aggressive disease to try and target these therapeutic strategies. Clinical predictors have probably been the most broadly studied. In both Crohn's disease (CD) and ulcerative colitis (UC), age at diagnosis, disease location and smoking habit are currently the strongest predictors of disease course. A younger age at onset is associated with more aggressive disease both in CD and UC. Disease location in CD is associated with different types of complications: surgery and recurrence in upper gastrointestinal and proximal small bowel disease; and surgery in distal small bowel disease and peri-anal lesions in rectal disease. In UC, extensive colitis is clearly been associated with more severe disease. Finally, active smoking globally increases disease severity in CD but decreases it in UC. Besides these important factors, others may predispose to some specific disease evolution and complications, and are also reviewed in the present paper.

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INTRODUCTION

The clinical course of the disease varies greatly among patients with inflammatory bowel diseases (IBD). It usually follows a pattern of more or less frequent relapses of variable duration interspersed with remission periods, but some patients may experience progressive decrease and even disappearance of disease activity over time, while others may have chronic active disease without remission periods. Furthermore, the natural history of IBD progressively leads to the development of complications in approximately two-thirds of Crohn's disease (CD) patients[1,2] and less than one-third of ulcerative colitis (UC) patients[3,4]. In CD, the main complications are the development of fibrotic strictures that lead to intestinal obstruction and the development of intra-abdominal and peri-anal fistulae and abscesses. In UC, the main complications are fulminant colitis that leads to emergency colectomy and the development of colorectal cancer.

To be able to predict the course and complications of IBD has become particularly important since drugs able to change the natural history of the disease have become available: this is particularly the case for anti-tumor necrosis factor treatment[5,6] but probably also, albeit to a

lesser extent, immunosuppressive treatment if it is used early enough[7].

Among other markers that can help clinicians to predict disease course in IBD, demographic and clinical characteristics are important because they are probably the easiest to use. The value of clinical parameters has been studied for the prediction of various types of disease outcomes, including the development of complications, need for surgery, or more elaborate definitions of disabling or severe disease.

CD

The variable course of CD has been well described in several Scandinavian population-based studies[8,9]. In particular, four subtypes have been described: 1, severe flare at diagnosis but with rapid decrease and disappearance of disease activity; 2, mild activity at the beginning with secondary progressive worsening of the disease; 3, recurrent flares interspersed with periods of full remission; and 4, chronic active disease with fluctuating but constant disease activity. In a recent population-based Norwegian study, types 1, 2, 3 and 4 affected 43%, 3%, 32% and 19%, respectively[9]. To date, no clinical predictive factor has been associated with these different profiles of disease evolution. More numerous studies have been devoted to the search for predictive factors of disease complications.

The characteristic of CD that is associated most strikingly with the course of the disease is disease location. Terminal ileal location has been very strongly associated with the risk of stricture and internal penetrating behavior in several independent studies[10,11]. Hence, it has also been associated with the risk of surgery[12,13]. Proximal small bowel and upper gastro-intestinal tract location (category L4 in the Montreal classification) has been associated in a European population-based study with the risk of recurrence[14]. In a recent Asian study, it was also associated with the risk of surgery[15]. Colonic disease, and particularly rectal disease, is associated with the development of peri-anal disease, and such a complication develops in 12% of patients with ileal disease, 41% of those with colonic disease, and 92% of those with rectal disease[16].

The behavior of the disease at diagnosis can also influence the course of the disease, since a fibro-stenosing or a perforating phenotype is already the sign of the presence of complications. By itself, a particular phenotype may necessitate surgery or the use of immunosuppressive drugs. Beyond this, the predictive value of the phenotype at diagnosis is more difficult to imagine. One of the reasons is that the phenotype of CD is a dynamic process, and after 10 years, almost 50% of the patients with an inflammatory phenotype at diagnosis develop fibro-stenosing or perforating complications that affect the course of the disease[1]. However, it must be remembered that, if only a minority of CD patients remain complication-free over time (30% at 10 years and < 20% at 20 years), among those developing complications, these will occur with a variable delay after diagnosis. This can be interpreted as a different inclination to develop such complications, that is, patients who have already developed complications at diagnosis are the most prone to develop such complications. This difference may then influence the course of the disease.

The presence at diagnosis of peri-anal lesions, is associated with a more frequent use of immunosuppressive treatment[17,18], poor mid-term outcome[19], and increased risk of surgical resection and postoperative recurrence[20]. A stricture or intra-abdominal penetrating lesion is associated with an increased risk of surgery[12,18]. Other studies have also shown that abdominal penetrating lesion at the time of first surgery is associated with more rapid recurrence and is an indicator of further surgery[21].

More recently, some studies have been performed in an attempt to define predictive factors for the development of disabling or severe disease over time. In a large cohort study that used retrospective analysis and prospective validation, the need for surgery, immunosuppressive treatment, biological agents, and hospitalization, demonstrated that peri-anal disease, together with young age at diagnosis and the need for steroids to treat the first flare, were factors associated with disabling chronic disease[22]. In a further study, these factors were partly confirmed, particularly the presence of peri-anal disease at diagnosis[23]. Furthermore, the presence of a stricture or penetrating disease at diagnosis, and peri-anal disease together with weight loss, fever and increased platelet count, were associated with development of severe disease, which was defined by surgical resection of two small bowel segments (or at least 70 cm) or any colonic segment, complex peri-anal disease, or definitive stoma[23]. However in a European population-based study, a stricture or penetrating behavior at diagnosis was not associated with recurrence after the first flare, need for surgery[14], or increased mortality[24].

Young age at onset is also associated with greater disease severity, particularly with the risk of disabling disease[22,23]. However, in contrast, older age at onset is associated with a slight increase in mortality[24].

As indicated earlier, weight loss at diagnosis is associated with more severe disease that is characterized by development of non-reversible tissue damage[23]. Obesity has been associated in a recent study with a more rapid need for surgery, which indicates a more aggressive disease in these such patients[25].

Finally, active smoking is associated with disease activity[26], as well as with the development of strictures and fistula complications[10,11,27]. In a recent study, low levels of smoking increased disease activity and hospitalization, but not the need for surgery[28].

UC

Disease course also varies greatly among patients with UC. In a recent population-based study from Norway, nearly one-fifth of the patients experienced no significant relapse over 10 years after diagnosis, while about half of

the patients had quiescent disease over the past 5 years[29]. In UC, disease location is associated most strikingly with disease course. In particular, extensive colitis is associated with increased risk of colectomy[30] and colon cancer[31], as well as a slight increase in mortality[32]. Colectomy rate reached 35% in extensive colitis, while it was < 20% in left-sided colitis and < 10% in proctitis[30]. The same is true for the risk of colon cancer, which reached 45% after 30 years of the disease in one early historic study, while it was around 30% in left-sided colitis and 10% in proctitis[31]. These risks have been shown to be much lower in most recent studies. It has gone down to 5%-10% for cancer, probably due to sustained control of inflammation and perhaps surveillance, but still with an increased risk in extensive colitis[33,34]. Likewise, in a recent population-based European study, the global risk of colectomy was 8.7% over 10 years[4].

In a recent study, patients with distal colitis were at increased risk of disease extension if they were younger at diagnosis or if they had sclerosing cholangitis[35]. Furthermore after disease extension, these patients were at increased risk of more aggressive disease with resistance to treatment. In another recent pediatric study, disease extension was strongly associated with risk of colectomy[36]. It was also the case, albeit to a lesser extent, for extra-intestinal manifestations.

In a recent Norwegian population-based study, age > 50 years at diagnosis was associated with decreased risk of colectomy[29].

Finally, smoking is associated with a less aggressive disease course in UC[37,38]. In a recent study from Hungary, it even decreased the risk of colectomy[39].

CONCLUSION

Clinical presentation markedly influences disease course in CD and UC. It probably currently represents the strongest predictor for further disease evolution and complications. In both CD and UC, the clinical and demographic characteristics most constantly associated with disease course are age at diagnosis, disease location, and smoking. Younger age at onset is associated with a more severe course in both diseases. In CD, proximal small bowel and upper gastrointestinal tract location is associated with risk of recurrence and surgery; small bowel disease with risk of surgery; and colonic disease, and particularly rectal disease, is associated with increased risk of peri-anal lesions. In UC, extensive colitis is associated with increased risk of colorectal cancer and colectomy. Finally, smoking globally decreases disease severity in UC while it increases severity in CD.

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TOPIC HIGHLIGHT

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Serologic and laboratory markers in prediction of the disease course in inflammatory bowel disease

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Abstract

The search for biologic markers that can assess the natural history and perhaps predict the course of individual's disease including response to treatments over time has become an important focus of inflammatory bowel disease research. The knowledge of an individual's prognosis can help physicians and patients make important management decisions and aid communication on risk and benefits of disease and treatment.

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Key words: Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; Perinuclear anti-neutrophil antibody; Anti-*Saccharomyces cerevisiae* antibody; Pouchitis; Internal penetrating; Fibrostenosing

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INTRODUCTION

The ideal serological and laboratory marker should be able to identify individuals at risk for the disease and should be disease specific. Moreover it should be able to detect disease activity and monitor the effect of treatment; and finally it should have a predictive value towards relapse or recurrence of the disease. Predicting disease course however has now been expanded beyond just disease recurrence, but perhaps more importantly to include predictors of disease complications including surgery. This topic highlight will focus on the prognostic role of serological and laboratory markers and the importance of phenotype stratification in inflammatory bowel disease (IBD) patients.

SEROLOGICAL IMMUNE MARKERS

The search for the underlying trigger of the abnormal intestinal inflammatory reaction characteristic of IBD has led to the discovery of antibodies present specifically in the blood of patients with Crohn's disease and/or ulcerative colitis (UC)[1-6]. Perinuclear anti-neutrophil antibody (pANCA) is noted for its association with UC or a UC like phenotype[7-13]. Anti-*Saccharomyces cerevisiae* antibody (ASCA) is another important antibody marker that is present in the blood of individuals with IBD[7-9]. ASCA is part of the family of anti-glycan (carbohydrate) antibodies. Other anti-glycan antibodies, antibodies against laminaribioside (ALCA) and chitobioside (ACCA) have been studied in IBD[14]. In addition to the anti-glycan antibodies, 3 additional markers representative of microbial driven immune responses have been identified; antibodies to the *Escherichia coli* (*E. coli*) outer-membrane porin C (OmpC), the *Pseudomonas fluorescens* CD related protein [anti-CD related bacterial sequence (I2)] and the CBir1 flagellin[15,16]. As seen with the genetic and clinical heterogeneity of CD, studies have shown immune response (immune phenotype) heterogeneity exists among

CD patients. Landers *et al*[15] analyzed immune response heterogeneity in 330 patients and demonstrated that CD patients could be clustered into 4 distinct groups depending on their immune response patterns to microbial or auto-antigens. One cluster was ASCA, a second was antibodies to OmpC and I2, the third pANCA and the fourth was low or no immune response to any tested antigens. Immune reactivity to CBir1 may further define CD phenotypes in that anti-CBir1 expression is present in 40%-44% of pANCA positive CD patients *vs* only 4% in pANCA positive UC patients. This difference may denote a unique etiopathogenic mechanism of disease that helps to further stratify patients based on immunogenetic phenotypes.

Vasiliauskas *et al*[17] introduced the notion of immune response stratification when he first reported that high ASCA levels were found to be associated with fibrostenosing (FS) and internal-penetrating (IP) disease as well as the need for small bowel surgery. Another cross sectional study demonstrated that patients who were ASCA IgA or IgG positive were 8.5 times and 5.5 times more likely to undergo early surgery (within 3 years of diagnosis) than ASCA IgA or IgG negative patients[18]. Mow *et al*[19] examined the association of multiple immune responses and disease phenotype. Reactivity to OmpC was independently associated with IP disease, while reactivity to anti-I2 was independently associated with FS disease and the need for surgery. Both the presence and magnitude of the immune response was associated with more aggressive disease behaviors. A similar study in a Scottish CD cohort reported that the cumulative reactivity to ASCA, I2 and OmpC was associated with small bowel complications[20]. Antibodies to CBir1 were examined in a later study and were found to be independently associated with small bowel disease, IP and FS disease[16]. Xue *et al*[21] demonstrated that reactivity to ASCA, OmpC and CBir1 was associated with early disease onset, FS and IP disease and the need for surgery. The presence and magnitude of the serological response to anti-glycan antibodies were found to be associated with complicated disease, disease duration, and need for surgery[22]. Subsequently, increasing amount and level of antibody responses toward gASCA, ALCA, ACCA, AMCA, and OmpC were found to be associated with more complicated disease behavior ($P < 0.0001$) and need for surgery in CD ($P = 0.023$) in an eastern European IBD cohort[23].

More recent pediatric cohort studies suggest that these markers are present in patients before a complication occurs and thus predictive of disease progression from uncomplicated to complicated disease state. Desir *et al*[24] demonstrated that baseline ASCA reactivity was associated with a more relapsing course in a pediatric CD cohort (OR = 2.9; 95% CI: 1.33-6.35). A multi-center study examined the association of ASCA, anti-I2, anti-OmpC, and anti-CBir1 reactivity with disease course in close to 800 pediatric CD patients[25]. The frequency of development of disease complications (IP and or FS) increased in parallel with reactivity to increasing numbers of antigens. The odds ratio for the development of IP disease was 5.0 and 9.5 for children with reactivity to 2 and 3 antigens, respectively. Furthermore, survival analysis demonstrated that reactivity to at least one microbial antigen was associated with the development of surgery faster as compared to patients negative for all markers suggesting that these markers may predict more aggressive disease behaviors. Amre *et al*[26] also studied a cohort of pediatric CD patients who had sera drawn at diagnosis and were studied for the subsequent development of complications of their disease. Survival analysis revealed that the time to first complication was more rapid for ASCA positive patients than those who were ASCA negative. Moreover the relative risk (RR) of a recurrent complication (RR = 3.68) and needs for an additional surgery (RR = 1.95) was significantly higher in ASCA positive patients. As compared to the positive association between ASCA, antibodies to OmpC, I2 and CBir1 and disease complication, pANCA has been shown to be associated with a more benign, UC-like disease course and negatively associated with small bowel complicating disease[17,27]. High pre-colectomy levels of pANCA (> 100 EU/mL) have been prospectively shown to be associated with the development chronic pouchitis all IBD patients undergoing IPAA[28]. More recently, the same group reported that anti-CBir1 may accelerate the development of chronic pouchitis in the face of high pANCA levels[29]. Melmed demonstrated that ASCA positivity and a family history of Crohn's disease was most predictive of CD of the pouch after IPAA[30]. This information may not change the need for colectomy but the surgical procedure chosen and the post operative management would certainly be impacted by this prognostic information.

FECAL CALPROTECTIN AND C-REACTIVE PROTEIN

Other biomarkers such as fecal calprotectin and C-reactive protein (CRP) have been examined as non invasive tools to predict disease course but with a focus on relapse or disease recurrence. Calprotectin is an abundant neutrophil protein found in both plasma and stool that is markedly elevated in infectious and inflammatory conditions including IBD. Calprotectin constitutes approximately 5% of the total protein and 60% of the cytosolic protein in human neutrophils[31]. Plasma calprotectin has been shown to increase 5 to 40 fold in infectious and inflammatory conditions[32] and fecal calprotectin concentration is approximately six times that of normal plasma[33]. The clinical course of most patients with IBD is typically marked by periods of remission with intermittent relapses. Because the timing of these relapses can be unpredictable, clinical symptoms more so than invasive tests are used to monitor disease activity. Most IBD relapses, however, come to medical attention after the inflammatory response has become well-established and clinical symptoms are worsening. Given that fecal calprotectin has been shown to correlate well with endoscopic and

histologic intestinal inflammation, its use as a predictor of relapse in patients with quiescent IBD has been examined. Tibble *et al*[34] studied 43 patients with CD and 37 patients with UC in clinical remission for 1-4 mo as determined by clinical disease activity indices and Harvey Bradshaw index. These patients had fecal calprotectin measurements at baseline and were then followed for 1 year. Approximately half of the patients in each group relapsed during the 12 mo follow up period. The sensitivity and specificity of calprotectin for predicting relapse within the year in all patients with IBD were 90% and 83%, respectively by using a cut off of 50 mg/L. This cut off was associated with a 13-fold increased risk of relapse. These values were similar when patients with CD or UC were examined separately. Costa *et al*[35] found nearly identical sensitivity and specificity of calprotectin for predicting relapse in UC, but their specificity of calprotectin in CD was 43%, markedly lower than the 83% reported by Tibble *et al*[34] noted above. This large discrepancy may be explained by the fact that their study used a different assay and a lower cut-off of fecal calprotectin (150 μg/g, corresponding to 30 mg/L). A more recent study prospectively evaluated 97 patients with UC and 65 with CD in clinical remission for at least 6 mo[36]. The cutoff level was set at 130 mg/kg of feces. Patients were followed up for 1 year after the test or until relapse. Fecal calprotectin was positive in 44 UC patients and 26 of them relapsed within a year, while 11 of 53 UC patients with a negative calprotectin relapsed within the year. Thirty CD patients had a positive calprotectin and 13 of them relapsed within a year, as did 7 of the 35 with a negative test result. A significant correlation emerged between a positive calprotectin and the probability of relapse in UC patients ($P = 0.000$). In CD patients, only cases of colonic CD showed a significant correlation between a positive calprotectin test and the probability of relapse, such that 6 colonic CD patients were positive for the calprotectin test and 4 relapsed ($P = 0.02$). A subsequent multicenter study demonstrated that fecal calprotectin concentrations in patients who suffered a relapse were higher than in nonrelapsing patients $P < 0.001$)[37]. Relapse risk was higher in patients having high (> 150 μg/g) calprotectin concentrations (30% *vs* 7.8%, $P < 0.001$). The area under the receiver operating characteristic curve to predict relapse using calprotectin determination was 0.73 (0.69 for UC and 0.77 for CD). Better results were obtained when only colonic CD disease or only relapses during the first 3 mo were considered (100% sensitivity).

CRP is one of the most important acute phase proteins in humans. Under normal circumstances CRP is produced by hepatocytes in low quantities but following an inflammatory stimulus, hepatocytes rapidly increase production of CRP under the influence of interleukin (IL)-6, tumour necrosis factor α, and IL-1β. CRP has a short half life (19 h) compared with other acute phase proteins and will therefore rise early after the onset of inflammation and rapidly decrease after the stimulus is resolved. Although CRP is upregulated in most inflammatory diseases, including IBD, CD is associated with a strong CRP response and UC only a modest and sometimes absent CRP response[38,39]. Moreover not all IBD patients mount a CRP response, even in CD and this must be kept in mind when measuring inflammatory markers in individual patients. It is unclear if this is due to differences in cytokine levels such as IL-6 or due to mucosal *vs* transmural disease differences among UC and CD or is genetically driven[40-43]. CRP has been shown to be a good marker for predicting disease course and outcome in a number of diseases with cardiovascular disease and poor outcome after myocardial infarction as well as in multiple myeloma[44-47]. Boirivant *et al*[48] prospectively followed 101 CD outpatients with 50% having raised CRP which correlated well with disease activity. However, 1/3 of patients presented with active disease despite normal CRP and 1/3 had raised CRP but reported inactive disease. The likelihood of relapse after 2 years was higher in CD patients with an increased CRP compared with patients with normal CRP. Perhaps the best study to date on the predictability of CRP and other biomarkers was reported by the GETAID group who prospectively followed 71 CD patients with medically induced remission and measured laboratory markers (full blood count, CRP, ESR, α1 antitrypsin, orosomucoid) every 6 wk[49]. In total, 38 patients relapsed after a median follow up of 31 wk. In the end only two laboratory markers were predictive of relapse: CRP (20 mg/L) and ESR (15 mm). Patients with both markers positive had an 8 fold increased risk for relapse with a negative predictive value of 97%, suggesting that normal CRP and ESR has a very high probability of ruling out relapse in the preceding 6 wk. There are fewer studies examining the role of these markers in predicting UC recurrence or outcome. One study evaluated 49 severe hospitalized UC patients treated with steroids and/or cyclosporine. On day 3, a stool frequency of > 8/d or 3-8 stools/d plus an increased CRP (0.45 mg/L) predicted with 85% certainty the need for colectomy[50].

CONCLUSION

Research has fostered a novel approach to understanding the intricate relationship between genetic and clinical expression of disease. Serum immune markers hold the most promise in helping researchers better comprehend disease heterogeneity and natural history. Although our current gold standard diagnostic tests do not possess this capability, prospective research studies now suggest that IBD-specific genetic and antibody markers may serve as predictors of an individual's disease course. Although promising at predicting disease recurrence, larger studies are needed with more frequent biomarker testing as this may allow for early detection of active, perhaps sub clinical inflammation which may alter treatment decisions. Their role as non-invasive predictors of post-operative recurrence is currently under investigation and will be of interest to help guide post-operative maintenance strategies.

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TOPIC HIGHLIGHT

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Role of genetics in prediction of disease course and response to therapy

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Abstract

The clinical course of Crohn's disease and ulcerative colitis is highly variable between patients, and this has therapeutic implications. A number of clinical features have been identified, which predict a mild or more severe outcome. However, several of these are subjective and/or not persistent over time. With the progress in genetics research in inflammatory bowel disease (IBD), genetic markers are increasingly being proposed to improve stratification of patients. Genetics have the major advantage of being stable over time and not prone to subjective interpretation. Nevertheless, none of the genetic variants associated with particular outcomes have shown sufficient sensitivity or specificity to have been implemented in daily management. Along the same line of thinking, pharmacogenetics or the study of association between variability in drug response and genetic variation has also received more attention as part of the endeavor for personalized medicine. The ultimate goal in this area of medicine is to adapt medication to a patient's specific genetic background and therefore improve on efficacy and safety rates. Although pharmacogenetic studies have been performed for all classes of drugs applied in IBD, few have generated consistent findings or have been replicated. The only genetic test approved for clinical practice is thiopurine S-methyltransferase testing prior to starting treatment with thiopurine analogues. The other reported associations have suffered from lack of confirmation or still need replication efforts. Nevertheless, the importance and necessity of pharmacogenetic studies will increase further as more therapeutic classes are being developed.

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Key words: Genetics; Inflammatory bowel diseases; Pharmacogenetics

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INTRODUCTION

The search for molecular markers to predict outcome in inflammatory bowel disease (IBD), has received much interest in the past years and is moving forward rapidly with the help of modern technologies. The recently completed and pivotal SONIC study as well as the Benelux Step-up/Top-down trial in Crohn's disease (CD) illustrated that treatment with biological agents early in the disease course results in higher steroid-free remission and mucosal healing rates, and impacts positively on the necessity of surgical resections[1,2]. However, from a clinical perspective, not all patients require thiopurine analogues and/or biological therapies. In addition, these

agents are not free from adverse events, including opportunistic infections and lymphomas. Predictors of a mild or complicated disease course would therefore help to select patients for a particular therapy.

GENETIC MARKERS TO PREDICT DISEASE COURSE

Compared to clinical parameters or serologic markers, genetic markers are more appealing for risk stratification as they are present long before the onset of the disease and before any environmental factor plays a role. In contrast to serologic factors, genetic factors are stable and are unaffected by disease flares. Moreover, as several studies have correlated microbial seroreactivity with genetic mutations in pattern recognition receptors, genetic markers may well prove to be superior[3,4]. Despite a large number of confirmed genetic variants in the susceptibility to CD and ulcerative colitis (UC), only very few have so far been proven to influence outcome. The presence of NOD2/CARD15 variants has been associated with a more rapid onset of stenosing small bowel disease and need for ileocecal resections[5-7]. In a Dutch tertiary multicentre cohort, Weersma *et al*[8] described a more severe disease phenotype, a higher need for surgery and a younger age at onset of CD with an increasing number of risk alleles and genotypes (NOD2, IBD5, DLG5, ATG16L1, IL23R). We studied whether the recently identified susceptibility genes for CD and their variants could improve stratification of patients, when applied together with other subphenotypes[9]. In a cohort of 875 CD patients with a median follow-up time of 14 years, homozygosity for the rs1363670 G-allele in a gene encoding a hypothetical protein near the *IL12B* gene was independently associated with a stricturing disease behavior with an odds ratio (OR) of 5.48 [95% confidence interval (CI), 1.60-18.83, $P = 0.007$] and furthermore with a shorter time to onset of these strictures ($P = 0.01$), and this was especially the case in patients with ileal involvement ($P = 0.0002$) (Figure 1). In the same cohort, male patients carrying a T-allele at rs12704036 T had the shortest time to development of non-perianal fistula. Presence of a C-allele at the CDKAL1 rs6908425 single nucleotide polymorphism (SNP) and absence of NOD2 variants were both independently associated with development of perianal fistula, particularly when colonic involvement and active smoking were present (Figure 2). Despite their potential promise, genetic markers will most likely never fully predict evolution of disease, because of the incomplete penetrance, their modest to low frequency and the role of other (environmental) factors in shaping the disease. The place of genetic markers in predicting disease outcome more realistically lies in their integration with other molecular markers, clinical data and environmental triggers (Figure 3).

GENETIC MARKERS TO PREDICT THERAPY OUTCOME

Prediction of response to therapy is as accurate as prediction of disease course, and will become even more important as more therapeutic classes are being developed. The success of genetic markers in predicting outcome to CD or UC therapy has been limited, in contrast to other fields such as oncology, where molecular markers have demonstrated clinical utility in predicting response to chemotherapy. The response to cetuximab, a monoclonal antibody to epidermal growth factor receptor in metastatic colorectal cancer is influenced by the KRAS mutation status, as the benefit of cetuximab seems limited to patients with KRAS wild-type tumors[10]. Likewise, germline mutations may also correlate with clinical outcome to chemotherapy. A subanalysis of a large phase Ⅲ study with bevacizumab (Avastin), a humanized monoclonal antibody to vascular endothelial growth factor (VEGF), in metastatic pancreatic cancer, showed that overall survival and progression-free survival were influenced by SNPs in the tyrosine kinase domain of the VEGF receptor-1[11]. As in almost all human diseases necessitating medical therapy, a variable response is also observed for most drugs used in IBD. Between 20% and 30% of patients are refractory to any given medication despite optimal dose and duration. Besides the response, side effects and toxicity are also variable. These factors are of course not all explained by genetics. Disease duration, severity, behavior (inflammatory or stenosing) and concomitant therapies may all influence the response to a drug. Among the genetic factors, genetic variations in drug metabolizing enzymes and target proteins, and also heterogeneity in the patient's genetic background will account for the variable response. Genetic polymorphisms in drug metabolizing enzymes will affect active drug concentrations and this, together with genetic polymorphisms of drug sensitivity (drug receptor genetic variants), will ultimately lead to heterogeneity in drug effects[12].

There are several reasons why pharmacogenetic research in IBD has witnessed only modest success: identifying molecular markers which influence the response to a drug is more difficult than, for instance, the study of genetic markers that influence toxicity. Side effects are usually easy to define and identify, in contrast to efficacy scores, which are often less well defined. In addition, and as already explained, treatment response in a heterogenic

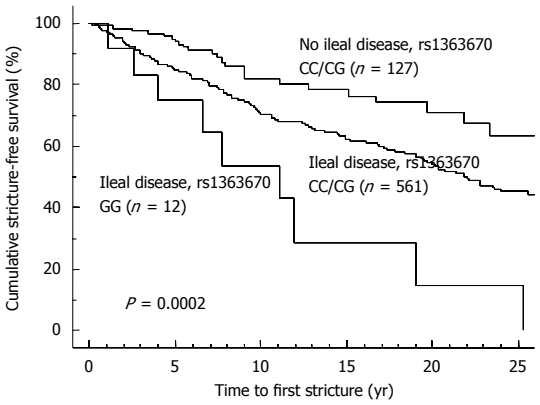


Figure 1 Time to onset of stricture formation in Crohn's disease patients.

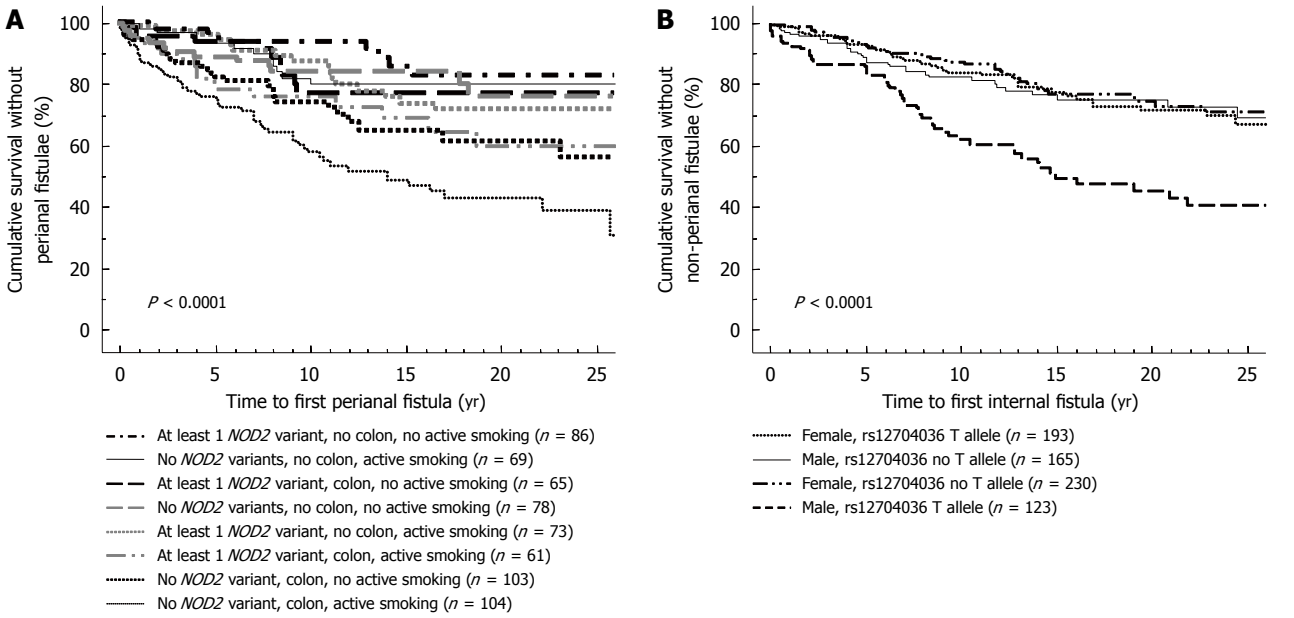


Figure 2 Stratification of patients with respect to development of perianal (A) or internal (B) penetrating disease.

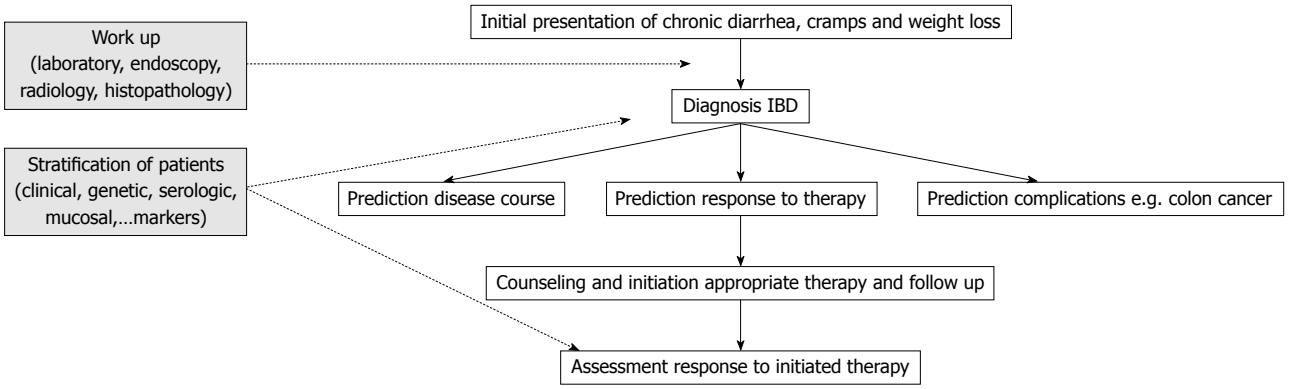


Figure 3 Implementation of genetic markers in management of inflammatory bowel disease (IBD).

disease like IBD is influenced by many factors such as disease duration, behavior and severity. Finally, besides polymorphisms in specific drug metabolizing enzymes or target proteins, heterogeneity in the patient's genetic background can also influence the response to treatment.

Since there are no or very little studies on genetic predictive factors for drugs such as methotrexate, cyclosporin, and tacrolimus, we focus in this overview on azathioprine and infliximab, on which most research has been conducted.

The only class of drugs where genetic testing for response prediction is useful and recommended are the thiopurine analogues. Azathioprine is metabolized by the enzyme thiopurine methyl transferase (TPMT), and the activity of this enzyme is under genetic control[13]. Mutations in the *TPMT* gene result in lower TPMT enzyme activity and this is associated with an increased risk for hematopoietic toxicity. In clinical practice, genotyping the most common TPMT variants or measuring TPMT enzyme activity can be carried out. Both techniques have advantages, and which technique is used is in part dependent on the availability. TPMT enzyme activity is measured in red blood cells with a radiochemical or high-performance liquid chromatography assay. The results can be influenced by blood transfusions but also other drugs may interfere with the TPMT enzyme activity (e.g. diuretics, 5-aminosalicylic acid). TPMT enzyme activity will identify patients with high TPMT activity that metabolize 6-mercaptopurine (6-MP) to 6-methyl-MP and therefore may be resistant to treatment with thiopurine drugs. Genotyping is an easier method, but genotypes do not fully correlate with the enzyme activity, especially in the case of wild-type (some patients will have reduced TPMT activity) or heterozygous (some will have a normal TPMT activity) individuals[14-17]. Therefore, measuring TPMT enzyme activity will give a more accurate picture. In patients in whom TPMT testing is performed, azathioprine or 6-MP can be initiated at normal doses (2.5 and 1.5 mg/kg, respectively) in the case of a wild-type genotype or normal TPMT enzyme activity. When TPMT activity is intermediate or when pa-

tients are heterozygous for the common TPMT variants, a dose reduction of 50% is recommended. Finally, low or absent TPMT activity and/or compound heterozygous/homozygous mutant patients should not be initiated on azathioprine or 6-MP given the high risk of myelotoxicity. Socioeconomic analyses showed that TPMT phenotyping or genotyping was cost effective[18]. However, hematologic toxicity can also develop in patients with a normal TPMT activity and this is the reason why monitoring of blood counts and liver transaminases remains necessary in all patients, as long as they are taking this drug.

Apart from TPMT, mutations in the *ITPA* gene (inosine triphosphate pyrophosphatase) have also been studied with respect to azathioprine toxicity. ITPAse deficiency would result in the accumulation of the potentially toxic 6-thio-ITP. Although some studies reported an association between the ITPA 94C>A deficiency-associated allele and azathioprine toxicity, other studies could not confirm this[19-21].

Corticosteroids (CS) are effective as induction therapy in moderate to severe active UC and CD. The long term outcome data look less promising, however, as European as well as North American studies showed that 25%-30% of patients became steroid-dependent and 20% steroid-resistant within 1 year[22,23]. CS are potent inhibitors of T cell activation and cytokine secretion and mediate their antiinflammatory effect through binding the intracellular glucocorticoid (GC) receptor (GRα). A homodimer of 2 activated GRs is translocated to the nucleus and this complex then binds to specific DNA sequences and controls the expression of target genes [e.g. inhibition of the *activator protein-1* (*AP-1*) gene and induction of the *IκBα* gene]. Several mechanisms have been proposed for the resistance to CS. Overexpression of the *MDR1* (multidrug resistance) gene and subsequent elevated p-glycoprotein-mediated efflux of the drug was the first[24]. Altered functions of the GR and an excessive synthesis of pro-inflammatory cytokines have also been suggested. Steroid-resistant asthma patients do not respond to high doses of inhaled CS but develop Cushingoid side effects. In these patients reduced peripheral T lymphocyte GR binding affinity and abnormalities of GR-AP-1 interaction and increased expression of GRβ (a truncated splice variant of the normal isoform GRα) are observed. GRβ is unable to activate steroid-responsive genes. Honda *et al*[25] reported GRβ mRNA expression in 83% of the patients with steroid-resistant UC compared to only 9% in steroid-responsive patients, and 10% in healthy controls and chronic active CD patients. These results were confirmed in a recent study from Japan, where the authors looked at the frequency of GRα and β positive cells in colonic biopsies of GC-sensitive (n = 6) and GC-resistant (n = 8) UC patients[26]. They also found that there were significantly more GRβ-positive cells in the GC-resistant group than in the GC-sensitive and the control groups. Whereas GRα mRNA was expressed in all UC patients, GRβ mRNA was expressed in only 1 patient in the GC-sensitive group and in 7 patients in the

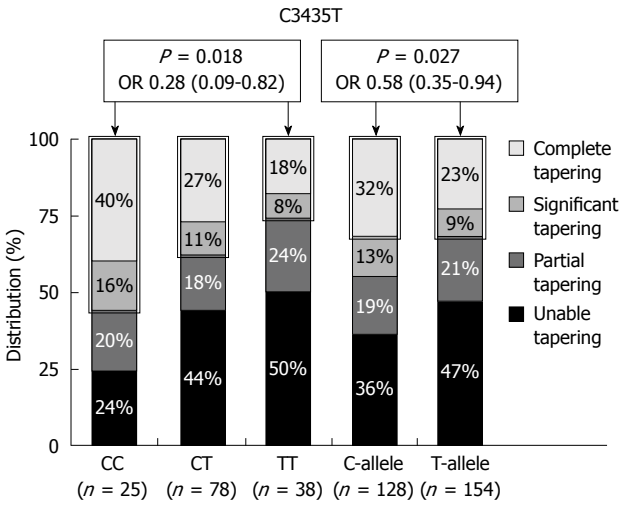


Figure 4 Distribution (%) of C3435T MDR1 genotypes and alleles with respect to glucocorticosteroid tapering in patients under azathioprine maintenance therapy[27].

GC-resistant group. Interestingly, the Foxp3+ cell count was also significantly higher in the GC-sensitive group.

The *MDR1* gene encodes for the drug efflux pump P-glycoprotein-170 (Pgp-170) and is expressed on the surface of lymphocytes and intestinal epithelial cells. Pgp and MDR expression were shown to be significantly higher in CD and UC patients requiring surgery due to failure of medical therapy[24]. MDR1 knockout mice spontaneously develop colitis and the MDR1 gene maps to the IBD susceptibility locus on chromosome 7 making it an excellent functional and positional candidate gene for susceptibility to IBD, and associations between C3435T and UC and G2677C/T and IBD have been described[27,28]. The MDR1 3435 TT genotype was especially associated with extensive UC. An association between steroid refractoriness and the 3435 TT genotype can therefore be the result of an extensive more severe disease phenotype in UC, but the TT genotype is associated with lower expression of MDR1 and Pgp170. Potocnik *et al*[29] reported an association between particular SNPs in introns 13 and 16 of the *MDR1* gene and CS-refractory CD and UC. The C3435T SNP in exon 26 was, on the other hand, associated with significant or complete CS tapering in a cohort studied from Leuven (Figure 4)[30]. Several other associations with SNPs in the *TNF* (tumor necrosis factor) gene and the macrophage *MIF* (migration inhibitory factor) gene and CS dependency or sensitivity have also been reported[31,32]. What is clearly needed from reviewing the literature on this topic, are trials in patient cohorts treated with fixed doses of CS and followed with well defined response criteria. Instead of looking for candidate genes, mucosal expression studies looking at differences between responsive and resistant patients may pave the way.

The use of monoclonal antibodies to TNF has greatly improved the quality of life of patients suffering from IBD but this therapy is expensive and not free from side effects. Although more than 75% of patients in general

respond to infliximab or adalimumab, resistance is seen in a subset of patients. If early response could be accurately predicted, management could be optimized.

Early studies looking at genetic variants with respect to anti-TNF outcome focused logically on the TNF and TNF receptor pathway. Specific mutations in these genes were studied but results were either negative, or positive results could not be confirmed in larger cohorts[33-35]. Since nuclear factor-κB signaling and TNFα levels are lower in cells carrying a CD-associated CARD15 variant it was also hypothesized that patients carrying a mutation in the CARD15 gene might respond differently to treatment with a TNF blocking agent. No significant associations were found also here in 3 independent cohorts of patients, including the ACCENT study cohort[36,37].

One of the mechanisms of action of infliximab is induction of apoptosis of monocytes and T lymphocytes[38,39]. It was therefore postulated that failure to induce apoptosis would be associated with lack of efficacy. In a small pilot study from Amsterdam, 14 patients were given infliximab and underwent endoscopy and 99mTc-annexin V single photon emission computer tomography scanning before and 24 h after infliximab administration (5 mg/kg)[40]. There was a clear and significant uptake of annexin V in the responders, whereas no such uptake was seen in the non-responders. This study showed that molecular markers may assist in predicting the outcome of anti-TNF treatment. Therefore, as the efficacy of infliximab is partly the result of the ability to induce apoptosis of activated T lymphocytes, we analyzed the effect of polymorphisms in apoptotic genes on outcome[41]. In luminal CD, there was a response rate of 74.7% in patients with the Fas ligand -843 CC/CT genotype compared with a response rate of 38.1% in patients with the TT genotype (OR, 0.11; 95% CI: 0.08-0.56, $P < 0.01$). The poorer outcome in patients carrying FasL-843T could be overcome by concomitant use of azathioprine. Patients with the caspase-9 93 TT genotype all responded, in contrast with 66.7% of patients with the CC and CT genotypes (OR, 1.50; 95% CI: 1.34-1.68, $P = 0.04$).

Also polymorphisms in the *Fcγ receptor 3A* gene, binding the Fc portion of the immunoglobulin have been studied, building on the hypothesis that infliximab leads to complement activation and antibody-dependent cellular cytotoxicity. The FcγRIIIa receptor 158V allotype displays a higher affinity for IgG1 and an increased ADCC and influences the therapeutic response to rituximab, an anti-CD20 IgG1 used in the treatment of non-Hodgkin lymphomas[42]. Similarly, Louis *et al*[43] showed an association between FCGR3A-158 and biological and possibly clinical response to infliximab in CD. However, this association could not be confirmed in a subset of 344 patients from the large and well-defined ACCENT 1 cohort of 573 patients[44]. There was however again a trend towards a greater decrease in C-reactive protein after infliximab in V/V homozygotes as compared with V/F heterozygotes and F/F homozygotes (-79.4, -76.5, and -64.3%, respectively, at week 6, $P = 0.085$; one-tailed $P = 0.043$). This finding has no immediate clinical impact but may enhance the understanding of the complex mechanisms of action of anti-TNF agents in CD.

More recently, microarray technology has been applied to advance our understanding on the reasons for (non)response to this class of agents. This technology enables measurement of the expression of thousands of genes simultaneously. In order to identify predictive gene profiles, we studied mucosal gene expression using the genome-wide Affymetrix HGU133 Plus 2.0 arrays in infliximab-naive CD and UC patients[45]. By comparing pre-treatment colonic mucosal expression profiles of responders with non-responders, we found that expression of IL-13Rα2 separated IBD responders from non-responders with 100% sensitivity and 91.3% specificity. The interleukin receptor, IL-13Rα2 has raised a lot of interest lately because of its involvement in fibrogenesis. IL-13 is a critical regulator of a Th2 response and is the key cytokine in parasite immunity and in the generation of an allergic response. IL-13 can bind to IL-13Rα1 with low affinity, causing downstream STAT6 activation. Alternately, IL-13 together with TNF-α can induce IL-13Rα2 expression and IL-13 can bind it with high affinity. This interaction leads to activation of the TGF-β1 promoter 20. It has been shown in a mouse model of colitis, that the initial Th1 response subsides after 3 wk and is followed by an IL-23/IL-25 response with IL-17 and IL-13 production, TGFβ1 production and the onset of fibrosis. If IL-13 signaling through this receptor is blocked by administration of soluble IL-13Rα2-Fc, or by administration of IL-13Rα2-specific small interfering RNA, TGF-β1 is not produced and fibrosis does not occur. Therefore, IL13Rα1 deserves further study.

Finally, for drugs such as methotrexate, cyclosporin, and tacrolimus, which are used less often in IBD, no or very few studies on genetic predictive factors have been conducted.

CONCLUSION

As physicians treating patients with IBD, the goal is to have at our disposal a molecular (serum, DNA, tissue-based) profile of our patient which will allow the most appropriate management (What is the most likely course of disease? Which is the most appropriate therapy with highest chances of success? Which should be the intensity of follow up?…). At the moment, only TPMT testing prior to start of thiopurine analogues has shown clinical applicability, but does not replace blood monitoring during treatment. Other reported genetic associations for the different therapeutic classes in IBD have not (yet) shown consistent or robust results. Studies looking at mucosal gene expression profiles could reveal novel pathways of (non)response. What is needed in pharmacogenetic and other predictive studies to advance the field are patients treated with standardized doses of the drug and fixed endpoints and criteria for response. The setting of a clinical trial may well be the preferred method for this and

attempts to collect DNA from these patients should be enforced.

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TOPIC HIGHLIGHT

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Mucosal biomarkers in inflammatory bowel disease: Key pathogenic players or disease predictors?

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Abstract

Inflammatory bowel diseases (IBDs) are chronic inflammatory disorders of the bowel, including ulcerative colitis and Crohn's disease. A single etiology has not been identified, but rather the pathogenesis of IBD is very complex and involves several major and minor contributors, employing different inflammatory pathways which have different roles in different patients. Although new and powerful medical treatments are available, many are biological drugs or immunosuppressants, which are associated with significant side effects and elevated costs. As a result, the need for predicting disease course and response to therapy is essential. Major attempts have been made at identifying clinical characteristics, concurrent medical therapy, and serological and genetic markers as predictors of response to biological agents. Only few reports exist on how mucosal/tissue markers are able to predict clinical behavior of the disease or its response to therapy. The aim of this paper therefore is to review the little information available regarding tissue markers as predictors of response to therapy, and reevaluate the role of tissue factors associated with disease severity, which can eventually be ranked as "tissue factor predictors". Five main categories are assessed, including mucosal cytokines and chemokines, adhesion molecules and markers of activation, immune and non-immune cells, and other mucosal components. Improvement in the design and specificity of clinical studies are mandatory to be able to classify tissue markers as predictors of disease course and response to specific therapy, obtain the goal of achieving "personalized pathogenesis-oriented therapy" in IBD.

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Key words: Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Disease course predictors; Cytokines; Chemokines; Intestinal mucosa

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INTRODUCTION

During the past decade, major insights into the pathogenesis of inflammatory bowel disease (IBD) have been achieved, and investigators generally agree that factors including the external environment, genetic makeup, intestinal microbial flora, and immune system are all involved and functionally integrated in the generation of

the chronic intestinal inflammatory reaction that characterizes IBD[1].

Among all factors, those considered to contribute mainly to IBD pathogenesis are genetics, along with mucosal immunity. New insights in mucosal immunity have allowed for the introduction of biological agents, which have led to great changes in clinical management of IBD, as well as the course of the disease.

Biological drugs block key molecules that are involved in the induction and maintenance of intestinal chronic inflammation through several pathways, differently represented among patients. However, although considered pathogenesis-oriented drugs, a lot still needs to be done in order to achieve "personalized pathogenesis-oriented therapy" in IBD. The complex pathogenesis that involves several major and minor contributors, involves different inflammatory pathways that can have diverse roles in different patients.

These components should be taken into consideration, when choosing from the variety of emerging therapies available for IBD patients, especially biological agents and immunosuppressants. These potent treatments may be associated with significant side effects, in particular serious infections and increased risk of malignancy. In addition, they might not be needed for the subgroup of patients destined to have a more benign course. Their high cost also requires optimal treatment-to-patient adjustment.

As a consequence, there is a pressing need to differentiate between patients who may have more aggressive phenotypes from those with a potentially more benign course[2], with the use of markers that are capable of predicting therapeutic responses.

Major attempts regarding the prediction of therapeutic efficacy have been made on clinical characteristics (e.g. smoking is associated with lower response rates[3]), concurrent medical therapy, serological markers (anti-Saccharomyces cerevisiae antibody and anti-neutrophil cytoplasmic antibody[4-6], outer membrane porin C, CBir1-flagellin, antibodies against I2 protein and the anti-glycan antibodies) and genetic markers[2]. All the above markers have been assessed for their response to anti-tumor necrosis factor (TNF)-α therapy. Emerging evidence, mostly related to the capacity of biological agents to induce mucosal healing, has suggested that mechanisms related to mucosal disease/healing are helpful in completing the scenario of predictors of specific therapy in IBD.

However, how mucosal and tissue inflammatory pathways can influence or predict the disease remains largely unknown, therefore making both prediction of disease course and clinical management of IBD a challenging task.

In fact, only few reports exist on how mucosal/tissue markers can predict clinical response, mostly related to infliximab treatment (summarized in Table 1 and briefly reported below).

Arijs *et al*[7] have shown that mucosal extracts that overexpress five genes, which encode osteoprotegerin (TNFRSF11B), stanniocalcin-1, prostaglandin-endoperoxide synthase 2, interleukin (IL)-13 receptor α 2 and IL-11, predict response to infliximab therapy with 89% accuracy in patients with refractory ulcerative colitis (UC). Notably, those genes encode for proteins involved in signaling in the adaptive immune response, inflammation and TNF-α pathways.

Another study from Van den Brande *et al*[8] has suggested that higher levels of apoptosis in the intestinal mucosa, as shown by 99mTc-annexin V uptake SPECT-CT images comparing before and after infliximab infusion, correlated with clinical response to infliximab in active Crohn's disease (CD) patients.

Schmidt *et al*[9] have demonstrated that, in 19 steroid-refractory CD patients treated with infliximab or cyclophosphamide, lower mucosal TNF-α concentration predicted long-term remission with a sensitivity of 100% and a specificity of 87.5%.

Similar to the study mentioned above, Olsen *et al*[10] have reported that an inverse association between pre-treatment TNF-α expression levels and clinical/endoscopic remission following infliximab treatment exists in UC patients with moderate to severe disease activity. In the same study, neither age, sex, steroid therapy, immunosuppression, pancolitis, endoscopic sub-score, disease duration, C-reactive protein, IL-4, IL-10 nor interferon (IFN) predicted mucosal or clinical remission.

Arsenescu *et al*[11] have revealed that, in a cohort of 69 CD patients, those with low levels of RelA subunit of nuclear factor (NF)-κB, A20 (a negative regulator of NF-κB), polymeric immunoglobulin receptor (pIgR), TNF-α and IL-8, had moderate to severe disease and poor responses to immunosuppressive and anti-TNF-α therapy, while patients characterized by low expression of RelA, A20, and pIgR, normal TNF-α and elevated IL-8, had acute inflammation that responded well to therapy.

Few reports are available on tissue factors as predictors of response to corticosteroids. Ishiguro[12] have found a higher protein level of IL-6 and TNF-α in biopsies from patients with intractable disease who were receiving corticosteroids compared to those with non-intractable disease receiving corticosteroids; these findings have not been confirmed in other studies[13].

Alternately, Raddatz *et al*[14] have found that, in patients with IBD, glucocorticosteroid receptor (GR) expression was not different from controls, although it was decreased in biopsies from UC patients who were non-responsive to glucocorticosteroids. In those patients, the inhibitory subtype GRb was expressed 100-1000 times less than GRa. On the contrary intercellular adhesion molecule-1 (ICAM-1) mRNA expression, although elevated in biopsies from UC patients with active disease, was no different in responders or patients with impaired response[14].

Another study has shown by univariate analysis that, in a total population of 561 UC patients evaluated with a median follow-up of 21.4 years since disease onset, mean histological inflammation and steroid use were predictors of colectomy[15].

Moving to tissue markers as predictors of disease

relapse, Yamamoto *et al*[16] have shown by multivariate analysis that higher rectal mucosal IL-8 level, younger age, and greater number of prior relapses were independent significant risk factors for future relapse in patients with quiescent UC. In the same study, IL-1, IL-6 and TNF-α levels in the rectal mucosa were not associated with relapse.

In light of these experiments, the aim of this paper is to review the role of tissue factors associated with disease severity, which may eventually be ranked as predictors of disease course and response to specific therapy. A schematic representation of the five proposed main categories of mucosal biomarkers, including mucosal cytokines and chemokines, adhesion molecules and markers of activation, immune and non-immune cells, and other mucosal components, is shown in Figure 1.

MUCOSAL CYTOKINES AND CHEMOKINES

Alterations in the production of many cytokines have been described in patients with active IBD[17]. Although the significance of these findings in the pathogenesis of IBD remains a controversial issue, as it is unclear whether they are primary or secondary defects in the regulation of the intestinal immune system, they can be considered as markers in the differentiation of groups of patients.

In active IBD, a disturbed balance between regulatory and effector cells has been described, which mainly involves effector T cells (Th1 and Th2) and regulatory T cells (Tregs, Th3). CD is associated with a Th1 T-cell cytokine profile, including IFN-γ, TNF-α and IL-12[18], whereas UC is associated with a modified Th2 type response cytokine profile including IL-15 and IL-10[18]. In addition, these findings have been recently complemented by the discovery of the IL-23/IL-17 axis that is part of the effector T cell immunological response, and seems to be involved in IBD. Levels of expression of IL-23 and IL-17 are increased in patients with active IBD[19].

TNF-α, IL-1, IL-6

IL-1 and TNF-α share a multitude of pro-inflammatory properties and are crucial in the amplification of mucosal inflammation in IBD. Both cytokines, primarily secreted by monocytes and macrophages, activate intestinal macrophages, neutrophils, fibroblasts, and smooth-muscle cells, inducing them to secrete prostaglandins, proteases, and other soluble mediators of inflammation and injury, as well as other chemotactic cytokines[20].

TNF-α is increased in the colorectal mucosa and stools of both forms of IBD[21,22], and correlates with a higher endoscopic inflammation score[23]. TNF-α is significantly increased in the non-inflamed mucosa of CD patients compared to controls[13,21], as well as in organ culture taken from macroscopically normal mucosa[24].

Other studies[13,22] have shown that several cytokines other than TNF-α are also upregulated in the intestinal mucosa of active IBD patients, including IL-1 and IL-6.

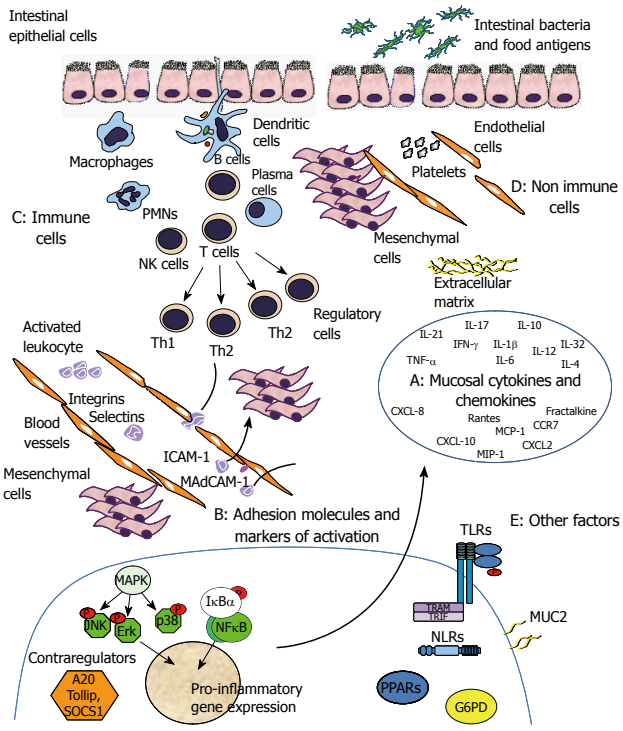


Figure 1 Mucosal biomarkers in inflammatory bowel disease (IBD). Based on available data from IBD pathogenesis, there are five major categories of mucosal biomarkers which can be assessed as predictors of disease severity and also response to therapy. A: Mucosal cytokines and chemochines [tumor necrosis factor (TNF)-α, interleukin (IL)-6, CXCL-2, *etc.*]; B: Adhesion molecules (MadCAM, ICAM-1, *etc.*) and intracellular markers of activation [mitogen-activated protein kinase (MAPK), nuclear factor (NF)-κB, A20, *etc.*]; C: Immune cells (dendritic cells, monocytes, macrophages, lymphocytes, NK, plasma cells, *etc.*); D: Non immune cells (endothelial cells, mesenchymal cells, platelets, *etc.*); E: Other factors [toll-like receptors (TLRs), NLRs, mucin (MUC), G6PD, *etc.*].

In addition TNF-α, IL-1β and IL-6 mRNA expression is increased compared to controls, with IL-6 being particularly high in the inflamed mucosa.

IL-4

IL-4 seems to play a crucial role in the Th2 response. One study has found that IL-4 is expressed in most biopsies from UC patients but not in CD[25], whereas others have not found any association between IL-4 levels and any form of IBD[13,26], probably due to technical issues[27].

IL-10

IL-10 is an anti-inflammatory cytokine produced by T cells, which inhibits the production of IL-1, IL-6 and TNF-α. Although a polymerase chain reaction (PCR)-based qualitative study on IL-10 in IBD has revealed that IL-10 is less frequently detected in UC compared to controls and CD[25], other studies have reported elevated mucosal IL-10 expression in UC and CD[28,29] or in UC alone[13,30,31].

IFN-γ

IFN-γ is elevated in all genetic animal models of IBD and is crucial in the development of Th1 responses.

Table 1 Studies assessing tissue markers as predictors of disease severity or drug response

Target	Techniques	Use	Ref.
Osteoprotegerin, STC1, PTGS2, IL13Rα2 and IL11	Microarray and PCR on mucosal extract	Predict response to IFX in UC	Arijs *et al*[7]
TNF-α levels and IL8, IL18	PCR and Immunohistochemistry on mucosal biopsies, ELISA on organ culture	Clinical/endoscopic remission in UC, response to corticosteroids, response to therapy in CD	Olsen *et al*[10] Ishiguro *et al*[12] Arsenescu *et al*[11] Schmidt *et al*[9]
RelA, A20, pIgR	PCR and Immunohistochemistry on mucosal biopsies	Severe disease and poor responses to immunosuppressive and anti-TNF-α therapy in 69 CD patients	Arsenescu *et al*[11]
GR expression	RT-PCR on mucosal biopsies	Decreased in biopsies from UC patients non responsive to glucocorticosteroids	Raddatz *et al*[14]
Mean histological inflammation and steroid use	Histology on colon biopsies	For univariate analysis, were predictors of colectomy in 561 patients UC	Hefti *et al*[15]
Mucosal IL-8 level	ELISA on mucosal biopsies culture	In multivariate analysis, independent risk factors for future relapse in patients with quiescent UC	Yamamoto *et al*[16]

GR: Glucocorticosteroid receptor; UC: Ulcerative colitis; CD: Crohn's disease; TNF: Tumor necrosis factor; IL: Interleukin; pIgR: Polymeric immunoglobulin receptor; ELISA: Enzyme-linked immunosorbent assay; RT-PCR: Reverse transcriptase polymerase chain reaction; IFX: Infliximab.

Mucosal IFN-γ expression is elevated in clinically active CD[32] but not in UC[33-35], which supports the theory that an imbalance in cytokine expression occurs in UC and CD; however, other studies have not reported such differences between UC and CD patients[36,37].

Other mucosal cytokines and chemokines

Other cytokines in the intestinal mucosa that are associated with active IBD include IL-21, IL-18, IL-8, monocyte chemotactic protein (MCP)-1, RANTES, epithelial neutrophil activating protein 78 (ENA-78), and IL-32[38].

Western blotting analysis of biopsies from patients with IBD and controls has demonstrated that IL-21 (a cytokine involved in the Th17 response) is overproduced in the inflamed intestine of patients with CD, in comparison to patients with UC and normal controls[39].

IL-18 is upregulated in intestinal mucosal cells of patients with IBD[40], and animal models have shown to benefit from anti-IL-18 antibody therapy with reduced severity in colitis[41].

In the proximal and distal regions of the colonic mucosa of UC patients, there was a greater than 10-fold increase in IL-8 levels compared to control subjects. A 3-5-fold increase in leukotriene B4 levels was observed along with a significant increase in myeloperoxidase levels throughout the colonic mucosa in patients with UC[42].

Other studies have reported elevated levels of MCP-1 and MCP-3, ENA-78, macrophage inflammatory proteins 1a and 1b, IFN inducible protein 10, stromal cell derived factor 1, and fractalkine[43-49].

Of particular interest is the demonstration that RANTES expression is not only increased in human IBD, but also plays a crucial role in the transition of acute to chronic disease in experimental models of colitis. In addition, RANTES triggers leukocyte adhesion to the inflamed intestinal microvasculature[50-52].

Furthermore, Kawashima *et al*[53] have observed a significant increase in CCR7 receptor only in CD and not in UC patients or healthy subjects. These observations are consistent with the reported increase in CC ligand expression in CD.

Studies assessing mucosal cytokine/chemokine panels

Several studies have attempted to explore the possibility of detecting tissue factors as predictors of disease severity by combining the analysis of different components simultaneously. However, none of these approaches has been validated and none are used for clinical purposes.

Real-time PCR quantification of CXCL8, CXCL10, calgranulin B and CXCL2 in colonic biopsies correlated with clinical activity index and endoscopic activity index in 27 UC patients, which suggests the use of these molecules as biomarkers and objective tools in clinical trials for the evaluation of anti-inflammatory and immunomodulatory regimens[54].

Another study with real-time PCR analysis (some confirmed by western blotting and enzyme-linked immunosorbent assay) of biopsy specimens from patients with UC and CD has shown increased expression levels of IFN-γ, TNF-α, IL-6, IL-15, IL-18, and IL-23 in affected and unaffected areas of IBD mucosa, compared to those found in healthy controls. Conversely, IL-1β, IL-6, IL-12, and IL-27 levels were higher in affected areas compared to unaffected ones in UC mucosa, but not in CD. A correlation between cytokine mRNA levels and inducible nitric oxide synthase and granzyme B has been observed[43].

ADHESION MOLECULES AND MARKERS OF ACTIVATION

Lymphocyte infiltration into the intestinal tract in IBD is mostly mediated by the interaction between α4β7 integrin expressed on lymphocytes and its specific ligand, mucosal vascular addressin CAM-1 (MAdCAM-1), which is expressed on the endothelial cells of the microvasculature in the inflamed intestinal tissue. Integrins form a large family of transmembrane proteins that are required for leukocyte-endothelium interaction, as well as cell-cell

interactions in the intestinal mucosa. These molecules together with the NF responsible for the signaling transduction pathway, such as NF-κB and mitogen-activated protein kinase (MAPK), are particularly abundant in the inflamed mucosa in UC and CD.

In one study[55], MAdCAM-1-expressing venules were more abundant in CD than in UC, whereas E-selectin was equally expressed in both diseases. Furthermore, CD was characterized by the presence of MAdCAM-1-expressing venules deep into the intestinal tissue, mainly localized in lymphoid aggregates.

Another group has reported[56] increased endothelial ICAM-1 expression in areas with dense lymphocyte infiltration, and areas close to crypt abscesses and ulcerations in the intestinal mucosa of active IBD patients. Ulcerations were covered by a continuous layer of macrophages and epithelial cells expressing ICAM-1.

We have reported previously[57] CD40 overexpression on mucosal endothelium in CD patients, which was reduced markedly after infliximab treatment, and reached a level similar to that seen in the normal mucosa of control subjects. In the same study, we reported vascular cell adhesion molecule-1 overexpression in the intestinal microvasculature of CD patients, which also disappeared after infliximab treatment.

Nikolaus *et al*[58] have shown that CD patients that relapse after induction of remission following infliximab administration, are characterized by increased TNF-α secretory capacity and mucosal nuclear NF-κB p65 before reactivation of clinical symptoms. Conversely, extended downregulation of nuclear concentrations of colonic mucosal NF-κB p65 is associated with sustained induction of remission.

Our group has reported increased phosphorylation of all three MAPKs (p38, extracellular signal-regulated kinase and Jun N-terminal kinase) in the microvasculature and mesenchymal cells of bowel preparations from patients with CD and UC, compared to controls and non-inflamed IBD mucosa[59]. In the same study, activation of these major signal transduction pathways was associated, *in vitro*, with increased production of inflammatory cytokines, as well as increased leukocyte adhesiveness to intestinal endothelial cells and fibroblasts.

IMMUNE CELLS

The uncontrolled inflammatory reaction in IBD is most likely the result of the interplay between genetic deficiencies in the innate immune system and an exaggerated T-cell-driven adaptive immune response[20].

IL-17-positive cells have been detected by immunohistochemical staining in the inflamed mucosa of CD and UC patients[60]. A recent study has confirmed the presence of Th17 cells in CD mucosa but the authors also found a previously unreported subset of mucosal T cells that share features with Th1 and Th17 cells (i.e. cells concomitantly producing IFN-γ and IL-17)[61].

Abnormalities in Tregs in human IBD have been described but are unclear because of the few inconclusive studies reported so far. It has been reported that, in the course of active UC and CD, the number of circulating Tregs is decreased compared to controls[62-64], but these differences do not persist during remission, which suggests that, in active IBD, Tregs migrate to the inflamed gut. In fact, paralleling the clinical activity of the disease, the number of Tregs during UC and CD has been reported to increase in the intestinal lamina propria and mesenteric lymph nodes[62,63,65-67]. One study from Japan has found that the relative proportion of CD4+CD25+ Tregs is significantly increased in patients with active IBD[68], whereas a German study has demonstrated that the frequency of CD4+CD25+ Tregs varies according to IBD activity[62]. In the latter study, Tregs were functional, but their number was reduced in the peripheral circulation and only moderately expanded in the inflamed mucosa.

Neutrophils and monocytic cells also play an important role in the pathogenesis of IBD and in the maintenance of active inflammation. Recently[69], infliximab has been reported to decrease histological disease activity in Crohn's ileocolitis, by lowering the number of neutrophils and mononuclear cells in intestinal mucosal biopsies. Furthermore, the number of lamina propria mononuclear cells is also reduced because of a global reduction in CD4(+) and CD8(+) T lymphocytes and CD68(+) monocytes.

Mucosal dendritic cells (DCs), the main antigen-presenting cells in the gut, display an activated phenotype in IBD tissues, which is indicative of their involvement in the local chronic inflammatory reaction. In particular, they have been shown to express elevated levels of CD40 in mucosal extracts from UC and CD patients. These levels decrease after patients are treated with anti-TNF-α[70]. Another group[71] has reported an imbalance in intestinal DC subpopulations in a group of pediatric CD patients that underwent surgical bowel resection, following different therapies. In particular, immature DCs (CD11c+CD83-CD68-DC-SIGN+) were only found in non-inflamed control colonic tissue, whereas mature (CD11c+CD83+CD68+DC-SIGN+) DCs were markedly reduced (60% and 30%) in CD tissue samples compared to controls. In addition, tissue samples from CD patients undergoing corticosteroid therapy display marked depletion of DCs compared to tissue from untreated patients or those treated with other drugs. Colonic tissue with severe inflammation shows reduced numbers of CD11c+ and CD83+ DCs in the lamina propria and submucosal compartments, with a concomitant increase in DCs in the muscularis, compared to moderately inflamed and non-inflamed CD tissue[71].

Active CD is also associated with an increased number of macrophages in the ileal and colonic mucosa. A recent study[72] has shown increased numbers of macrophages expressing the scavenger receptor CD163 in colonic mucosa of CD patients concomitantly affected by spondyloarthritis. Macrophages or DCs that express RANK (receptor activator of NF-κB ligand), CD68 and S100

protein are increased in the colon of CD patients, particularly in inflamed areas compared to normal colon[73].

NON-IMMUNE CELLS

Other important cell types that participate in the chronic inflammatory response of IBD, include epithelial, mesenchymal and endothelial cells, and platelets, which exert many of the functions traditionally attributed to classical immune cells, such as cytokine production or expression of major histocompatibility complex class Ⅱ antigens[18].

Immunohistochemical studies have shown that intestinal epithelial cells (IECs) inappropriately express the class Ⅱ antigen HLA-DR in actively inflamed mucosa of UC and CD patients[74]. More recently, IBD IECs have been reported to inappropriately express members of the B7 family of costimulatory molecules[56], a finding that suggests alterations in B7-ICOS costimulatory pathways in IBD.

Other studies have shown that, in IECs, expression of toll-like receptor (TLR)3 is significantly downregulated in active CD but not in UC, whereas TLR4 is strongly upregulated in both CD and UC[75]. In the mucosa of patients with postcolectomy active pouchitis, expression of TLR2 and TLR4 is also strongly upregulated[76]. In addition, patients with CD show a reduced content of defensins in the affected ileum, and this reduction is independent from the degree of mucosal inflammation[77].

The involvement of fibroblasts in IBD traditionally has been viewed as being restricted to the production of extracellular matrix components and the pathogenesis of a common and serious complication, such as intestinal fibrosis[78]. However, fibroblasts are also involved in gut injury because they represent a major source of matrix metalloproteinases (MMPs), a family of proteolytic enzymes that are directly responsible for the tissue destruction that occurs during inflammation.

In the course of IBD, upregulation of MMPs occurs in the intestinal tissue, and this increase is lowered significantly by the anti-TNF-α antibody infliximab. These observations have been confirmed by the work of Meijer *et al*[79]. They have reported increased expression of MMP and tissue inhibitor of metalloproteinase (TIMP) in the intestinal mucosa from patients with IBD, compared with control samples. Furthermore, infliximab downregulated expression of MMP-1, -3 and -9 but not TIMP-1 and -2, depending on genotype.

Wang *et al*[80] have confirmed these findings. They have shown that mRNA and protein expression of MMP-1 and TNF-α in the ulcerated and inflamed colon mucosa of patients with UC is significantly higher than that in non-inflamed mucosa of normal controls. Furthermore, the expression of MMP-1 and TNF-α in the ulcerated area is significantly higher than that in the inflamed area of patients with UC.

Endothelial cells play an essential role in inflammation as a result of their central gatekeeper function, which controls the quality and quantity of leukocytes that transmigrate from the vasculature into the interstitial space. This process is complex and is mediated by a number of molecules, including cytokines, chemokines and adhesion molecules.

Bardin *et al*[81] have shown that, in active disease, high expression of CD146 is observed on endothelial cells in intestinal biopsies from patients with CD and UC. In addition, there is a decrease in the soluble form of CD146 in relation to active disease and extent of CD and UC. Lower levels of soluble CD31 have also been detected in UC, but not in CD. This suggests CD146 as a novel marker of the endothelial intercellular junction, which reflects endothelial remodeling more effectively than soluble CD31 does.

Angiogenesis has been suggested to play an integral part in IBD pathology[82]. Vascular endothelial growth factor (VEGF) has long been considered to play a central specific role in angiogenesis. Our group has reported[82] increased microvessel density in IBD mucosa, with an increase in αVβ3 expression on new mucosal vessels. These observations are associated with increased mucosal levels of VEGF, which in turn are able to induce increased angiogenesis in corneal and chorioallantoic membrane assays. Other groups[83] have confirmed our findings, and have shown increased VEGF expression in CD and UC compared to controls. Furthermore, immunohistochemical staining for CD146 is also higher in both forms of IBD, as well as CD34 expression, which indicates a possible role of angiogenesis in the pathogenesis of IBD.

Endothelial junction adhesion molecules, such as CD146, recently have been suggested to play a role in angiogenesis. CD34 is expressed on the vascular endothelium, and is upregulated in IBD.

In active CD and UC, a loss in the expression of endothelial EPCR (the receptor for protein C) and thrombomodulin has been reported, therefore impairing the activated protein C pathway in the intestinal microvasculature[84].

OTHER MUCOSAL COMPONENTS

A positive correlation has been found between mucosal levels of total lactate dehydrogenase (LDH) isoenzyme M monomers and LDH 5 monomers for each of the histological subgroups of UC, while glucose-6-phosphate dehydrogenase activity seems to be independent of inflammatory changes[85].

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily of transcription factors whose activities are regulated by high affinity binding of small lipophilic ligands such as steroid hormones. PAR2 methylation status in rectal mucosa has been shown to correlate with more severe disease phenotypes of UC[86].

Mucin 2 (MUC2) precursor biosynthesis and total MUC2 levels are significantly decreased in UC patients with active inflammation, compared to controls. In contrast, both these parameters returned to control values

during remission from inflammation, which demonstrates that colonic biosynthesis and total amounts of MUC2 vary according to disease activity. However, *MUC2* mRNA levels are similar in all patients and are independent of disease activity, which indicates that these variations in MUC2 synthesis are regulated post-transcriptionally[87].

Immunohistochemical analysis[88] of cryostat sections, using polyclonal and monoclonal antibodies specific for TLR2, TLR4 and CD14, have shown a significant increase in TLR2 expression in the terminal ileum of patients with inactive and active UC compared with controls. Significant upregulation of TLR4 expression relative to controls has been found in the terminal ileum and rectum of UC patients in remission and in the terminal ileum of CD patients with active disease. CD14 expression is upregulated in the terminal ileum of patients with active and inactive CD. It is also upregulated in the cecum of UC patients in remission and with active disease, and in the rectum of patients with active UC.

CONCLUSION

The identification of tissue markers, together with other predictors, is crucial for clinical assessment and management of IBD. Although several attempts have been made, it is clear that more efforts should be made in creating a wider analysis, which involves more IBD centers and patients. To achieve this, a prospective observational study to identify precursors for each subgroup of patients is necessary. More consistent data from immunohistochemistry or western blotting of cytokines on mucosal extracts are needed. Interesting data also can be derived from tissue indicators of activation, such as NF-κB, MAPK or markers of other pathways, which are also involved in IBD pathogenesis. Finally identifying whether an immune or non-immune cell type is involved in mucosal inflammation through specific markers of activation/function, also represents another important aspect to be addressed by further research.

In conclusion, we must admit that, to date, despite great potential, no tissue factor can be considered as a predictor of disease progression or response to therapy in everyday clinical practice devoted to the management of patients with IBD.

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TOPIC HIGHLIGHT

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Role of endoscopy in predicting the disease course in inflammatory bowel disease

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Abstract

Endoscopy provides a direct evaluation of mucosal lesions in inflammatory bowel disease (IBD), permitting the description of elementary lesions, their surface extent and severity. The severity of mucosal lesions directly reflects disease activity and may help to identify an aggressive behavior of the disease. Several studies have recently pointed out the potential role of endoscopy in the prediction of IBD outcome. Indeed, severe endoscopic lesions in Crohn's disease (CD) patients, defined by deep and extensive ulcerations on at least one part of the colon, are associated with an increased risk of penetrating complication and surgery. Severe endoscopic lesions during severe attacks of ulcerative colitis (UC) are associated with an increased risk of colectomy in the short and long term. Severity of postoperative recurrence in CD may help to predict the risk of clinical relapse and need for further surgery. Achievement of mucosal healing, which can be obtained by administration of several types of drugs, is associated with a better outcome, less surgery and hospitalization. This review focuses on the assessment of endoscopic severity in CD and UC and on the impact of endoscopic severity on disease outcome. More specifically, we discuss how endoscopy can be used at different stages of IBD to predict the disease course and/or to adapt treatment strategies.

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Key words: Crohn's disease; Ulcerative colitis; Inflammatory bowel disease; Colonoscopy; Natural history; Outcome; Medical therapy; Surgery

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INTRODUCTION

The management of inflammatory bowel diseases (IBD) has dramatically changed over the last decade. Progress has been supported by the increasing size of the therapeutic arsenal, the introduction of biologics providing more alternative options in patients with severe diseases, and new concepts as to how and when treatments should be used. Immunosuppressants and biologics are classically used following a step-up approach in patients with refractory disease, who are unresponsive to conventional therapies or are steroid-dependent. Beyond their high efficacy in induction and maintenance of remission, it has been demonstrated that anti-tumor necrosis factor (TNF) therapies can close fistulae and heal mucosal lesions, and reduce rates of hospitalization and surgery[1]. Several recent studies suggest that early intervention with combination therapy may modify the long-term course of Crohn's disease (CD)[2-4]. However, these strategies should

not be used in all patients, but only in selected patients with severe diseases and/or with features predicting a poor prognosis[1]. Good predictors of IBD outcome are still lacking in our daily practice. Studies performed with regard to prediction have mainly focused on clinical and biological parameters. Endoscopy has been rarely investigated as a predictor of the clinical course of IBD. However, there is now growing evidence that morphological examination, including endoscopy, may help to identify among IBD patients those who should be treated with more intensive treatments. Furthermore, as demonstrated in a recent study assessing early intervention with combination of infliximab and azathioprine in CD, endoscopy may help to select patients who will obtain the best results with early intervention[2].

Endoscopy allows the examination of mucosal lesions in IBD[5,6]. The severity of mucosal lesions directly reflects the activity of the disease and may thus be considered as an indication of an aggressive behavior of the disease. In this article, we review aspects of assessment of endoscopic severity in CD and ulcerative colitis (UC) and discuss their role in predicting the course of these diseases and their impact on decisions regarding medical strategies.

ASSESSMENT OF ENDOSCOPIC SEVERITY IN CD

Endoscopic scores of severity in CD

A number of mucosal characteristics may be observed at endoscopy in CD, such as erythema, swelling, nodularity, strictures, aphtoid ulcerations and ulcers of variable size and depth. The severity of ileocolonic lesions can be quantified by validated endoscopic indices of disease activity. The CD endoscopic index of severity (CDEIS) is based on the recognition of elementary lesions (non-ulcerated lesions, superficial and deep ulcerations), associated with the appreciation of their surface in five segments (ileum, right colon, transverse, left colon and sigmoid, and rectum)[7,8]. A simplified index, the simple endoscopic score for CD (SES-CD), has been proposed and correlates well with the CDEIS[9]. The CDEIS is considered as the gold standard for quantifying endoscopic severity, but is often regarded as not easy to use. It requires a specific training for the estimation of ulcerated or diseased mucosal surfaces.

These scores can be used to assess endoscopic severity during active phases of the disease, and they may also be used to assess changes induced by medical treatments. Several studies on mucosal healing have been performed in CD. However, there is no validated definition of mucosal healing in CD[10]. The ideal definition is absence of ulcerated lesions. However, this definition may be excessively strict. For example, a patient who had deep and extensive ulcerations at index colonoscopy and who healed almost all lesions but still had one aphtous ulcer would not be considered as achieving complete mucosal healing. For this reason, we think that mucosal healing should be evaluated through validated scores on endoscopic severity.

Severe endoscopic lesions in CD

It is widely accepted that severity of colonic lesions in CD relies on the extent in depth and in surface of the mucosal damage. Recognition of these lesions by an endoscopist may be seen as subjective. However, it appears that diagnosis of severe endoscopic lesions may not be difficult. A previous interobserver variation study targeted on evaluation of ileocolonoscopic lesions in CD[11] has shown that deep ulcerations and estimation of ulcerated surface were among the most reproducible endoscopic items. Such lesions were also selected by multivariate analysis for the construction of the CDEIS[7].

Nahon *et al*[12] demonstrated that colonoscopy accurately predicts the anatomical severity of colonic CD attacks. In this retrospective study of 78 patients operated for colonic CD resistant to medical treatment, criteria of severity in colectomy specimens were defined as either deep ulcerations eroding the muscle layer, or mucosal detachments, or ulcerations limited to the submucosa but extending to more than one third of one defined colonic segment (right, transverse, left colon). Three endoscopic criteria of severity were defined: (1) deep ulcerations eroding the muscle layer; (2) deep ulcerations not eroding the muscle layer but involving more than one third of the mucosal area; and (3) mucosal detachment at the edge of ulcerations. Evaluation of endoscopic severity correlated well with findings on colectomy specimens. At least one of these criteria was found in 95% of patients with severe anatomic lesions on colectomy specimens. The extent of ulcerations at colonoscopy was correlated to the results of colectomy specimen examination ($P < 0.001$). Of note, usual clinical and biological severity criteria were not different in patients with severe colonic lesions or without. This study further demonstrates that colonoscopy can accurately assess anatomical severity of colonic CD.

THE ROLE OF ENDOSCOPY IN PREDICTING THE COURSE IN CD

Severe endoscopic lesions predict a more aggressive outcome with increased rates of complications and surgery in CD

Early studies by the Groupe d'Etudes Thérapeutiques des Affections Inflammatoires du Tube Digestif (GETAID) have shown that endoscopic pattern severity (assessed by the CDEIS) correlated poorly with clinical (assessed by the CDAI) and biological activity (assessed by C-reactive protein measurement)[8]. Furthermore, clinical improvement is not always associated with healing of mucosal lesions in CD. Indeed, clinical remission obtained with steroids is associated with mucosal healing in only one third of CD patients[11]. Moreover, the degree of endoscopic improvement in patients with prednisolone-induced clinical remission did not predict

the subsequent clinical course in terms of steroid weaning, or occurrence of relapse[13].

However, endoscopic severity may still have an impact on the long term course of the disease. We have shown in a retrospective study that patients with CD exhibiting deep and extensive ulcerations at colonoscopy have a more aggressive clinical course with an increased rate of penetrating complications and surgery[14]. Among the 102 patients included, 53 had severe endoscopic lesions at index colonoscopy, defined as extensive and deep ulcerations covering more than 10% of the mucosal area of at least one segment of the colon. During the follow-up (median 52 mo), 37 patients underwent colonic resection. Patients with severe endoscopic lesions needed significantly more colonic resections than patients without severe lesions (relative risk 5.43, 95% CI: 2.64-11.18)[14]. Six patients had penetrating complications during the follow-up. All had severe endoscopic lesions at index colonoscopy, performed in most of them several years before. These data suggest that a subset of CD patients have a more aggressive disease, characterized by severe endoscopic lesions in the ileocolon during symptomatic phases, and a higher risk of surgery and penetrating complications[14].

Mucosal healing and long term outcome in CD

In clinical trials, the efficacy of therapies in CD is usually assessed by improvement in clinical activity (defined by a decrease of the CDAI). Assessment of endoscopic improvement was not usually performed until recently in clinical trials assessing the efficacy of drugs in CD. The main reason for this was that steroid-induced clinical remission is not associated with mucosal healing in two-thirds of CD patients. Furthermore, endoscopic remission obtained with steroids does not correlate with the risk of relapse after steroid withdrawal. However, there is growing evidence that mucosal healing during therapy is a sign of a good efficacy of a drug[10,15]. Data from the IBSEN cohort strongly suggests that mucosal healing predicts a generally favorable outcome of disease based on all types of treatment strategies (except corticosteroids) and is related to treatment efficacy, reduced frequency of surgery and hospitalizations[16].

Moreover, it is now clearly demonstrated that mucosal healing can be achieved with azathioprine and anti-TNF[17-21]. Rates of mucosal healing under azathioprine vary among studies, probably due to differences in the timing of endoscopy and the population analyzed. In a randomized controlled trial performed in steroid-dependent CD patients, Mantzaris *et al*[22] have recently shown that azathioprine was superior to budesonide in inducing mucosal healing at 1 year; complete or near complete healing was achieved in 83% of azathioprine-treated patients compared with only 24% of budesonide-treated patients ($P < 0.0001$). In a GETAID study, long lasting remission ($\geq$ 42 mo) maintained with azathioprine was associated with a complete mucosal healing (CDEIS = 0) in only 36% of CD patients[23]. In the SONIC study, which concerned CD patients naïve to immunosuppressants and biologics, only 15.6% of patients treated with azathioprine achieved mucosal healing at week 26[2].

In the endoscopic substudy of the ACCENT I study, patients treated with scheduled maintenance therapy with infliximab had superior rates of mucosal healing, and those who maintained complete mucosal healing over 1 year had a lower rate (but the difference was not significant) of hospitalizations and surgeries[24,25]. A study of mucosal healing in a cohort of CD patients under long-term treatment with infliximab was recently reported[26]. In this study from the Leuven group, 214 patients had a colonoscopy before and a second one within months after starting infliximab. Mucosal healing (complete or partial) was observed in 68% of the 183 initial responders. Mucosal healing was associated with a significantly lower need for major abdominal surgery during long-term follow-up (14.1% major surgeries in patients with mucosal healing *vs* 38.4% in patients without mucosal healing, $P < 0.0001$). Interestingly, partial mucosal healing had the same favorable impact as complete mucosal healing.

Several studies suggest that immunosuppressants and anti-TNF therapy may be more effective when given early in the course of the disease. Two studies have been recently performed in CD patients naïve to immunosuppressants and biologics, including "top-down strategy" which refers to early introduction of immunosuppressive or biologic therapies. In the SONIC study, infliximab therapy was superior to azathioprine in inducing mucosal healing at week 26 (30.1% *vs* 15.6%), but inferior to infliximab plus azathioprine combination therapy[2]. D'Haens *et al*[3] compared a top-down strategy to a more classical step-up strategy. Top-down strategy, which consisted of early induction with infliximab and maintenance with azathioprine, resulted in mucosal healing in 19/26 of patients (73%) at week 104. In the other arm (step-up strategy), mucosal healing was significantly less frequent (7/23 patients, 30%). This difference may have an impact in the long term. Indeed, when mucosal healing was achieved at 2 years (SES-CD score at 0), 70% of the patients (17/24) were in stable clinical remission during the following 2 years as compared to only 6 of the 22 (27%) who had mucosal lesions (SES-CD score above 0)[4]. Fifteen of the 17 patients with mucosal healing at year 2 maintained in remission without further infliximab infusions during the following 2 years. Mucosal healing obtained with immunosuppressants or anti-TNF agents was also associated with a decreased risk of surgery in the long term. Altogether these data would suggest checking endoscopic response in patients treated with immunosuppressants or anti-TNF. However, this attitude would make sense only if we are ready to optimize or switch to another treatment those patients who have no mucosal healing. Another question which is raised by recent studies is whether partial mucosal healing is sufficient to modify the disease outcome with decreased risk of hospitalizations, surgery and complications.

A further question is whether mucosal healing under a therapy is associated with a decreased risk of relapse after

discontinuation of this therapy. In a placebo-controlled study of the GETAID, presence of ulcerations at ileocolonoscopy before withdrawal of azathioprine was not predictive of the risk of relapse[23]. A recent study from the GETAID assessed the risk of relapse after infliximab discontinuation in patients in remission on combined maintenance therapy, who continued the immunosuppressant (azathioprine or methotrexate). Mucosal healing was among the factors strongly associated with a decreased risk of relapse[27].

Endoscopy in predicting relapses of CD after surgery

CD patients who have "curative" ileal resection and ileocolonic anastomosis are exposed to a high risk of postoperative recurrence[28]. Rutgeerts *et al*[29] demonstrated in 1990 that ileocolonoscopy performed within 1 year of surgery may predict the risk of clinical recurrence. Eighty-nine patients treated by ileal resection for CD were included in this prospective cohort follow-up to study the natural course of early postoperative lesions. Within 1 year of surgery, ileocolonoscopy detected recurrent lesions in the neo-terminal ileum in 73% of the patients, although only 20% had a clinical relapse. The rate of clinical relapse was 34% at 3 years. A score was devised to assess the severity of recurrent endoscopic lesions. The course of the disease was best predicted by the severity of the early postoperative lesions, as observed at ileocolonoscopy, on the anastomosis and/or on the neo-terminal ileum. Indeed, patients with less severe endoscopic lesions according to Rutgeerts' score (less than 5 aphtoid ulcers at anastomosis site), have a lower risk of clinical recurrence risk at 9% compared with 100% risk at 4 years for patients with more severe endoscopic recurrence (Rutgeerts' score i2 or greater). This score is widely used in clinical practice, and ECCO guidelines state that ileocolonoscopy should be the gold standard in the diagnosis of postoperative recurrence by defining the presence and severity of morphologic recurrence and predicting the clinical course. Ileocolonoscopy is recommended within the first year after surgery where decisions of postoperative treatment may be affected[30].

ASSESSMENT OF ENDOSCOPIC SEVERITY IN UC

Endoscopic scores of severity in UC

Nine different scores have been proposed to measure endoscopic severity in UC. Some of these scores focus on the severity of bleeding, but most of them rely on the recognition of elementary lesions such as abnormal vascular pattern, granularity, friability, bleeding and ulcers[10].

Endoscopic severity in severe attacks of UC

Carbonnel *et al*[31] demonstrated that total colonoscopy is feasible in 86% of cases in severe UC (73/85). In this study, endoscopy accurately identified severe endoscopic lesions (extensive deep ulcerations). Eighty-five consecutive patients with attacks of UC were reviewed. Extensive deep colonic ulcerations were diagnosed in 46 of them. No complication related to colonoscopy occurred except for one colonic dilatation. Forty-three of the 46 patients with severe endoscopic colitis underwent surgery. Extensive ulcerations reaching at least the circular muscle layer were found on pathological examination and were confirmed in 42/43[31]. Because of potential risks of complications, some rules have to be applied when performing colonoscopy in patients presenting severe attacks of UC, including pre-radiological examination to exclude megacolon, minimal insufflations, and when severe lesions are detected, the examination can be stopped as further examination has no additional prognostic value.

THE ROLE OF ENDOSCOPY IN PREDICTING THE COURSE OF UC

Patients with severe endoscopic lesions during severe attacks of UC have an increased risk of colectomy

Among patients hospitalized for a severe attack of UC, the presence of extensive and deep ulcerations at colonoscopy is associated with an increased risk of colectomy on that admission[31]. In their study performed in the prebiologic era, Carbonnel *et al*[31] showed that colectomy was performed in 43 of the 46 patients who presented severe endoscopic lesions (93%) as compared to 10/39 (26%) of those without such lesions (OR 41). In another study performed in severe UC patients, severe endoscopic lesions at colonoscopy were significantly more frequent in non-responders to medical treatment (91%) compared with responders (34%) (OR > 20)[32].

The colonoscopies performed during severe attacks of UC have also an impact on the long term outcome, with an increased rate of surgery in the long term in patients who exhibit extensive and deep ulcerations at index colonoscopy[33].

Mucosal healing and outcome in UC

Mucosal healing is classically assessed in patients treated for UC. However, there is no consensus on the definition of mucosal healing in UC[10]. The International Organization of IBD proposed the following definition: absence of friability, blood, erosions and ulcers in all visualized segments of the gut mucosa. According to this definition, disappearance of the normal vascular pattern is compatible with mucosal healing.

Mucosal healing may be obtained with several types of drugs[10,34]. It has been shown that it can be obtained with 5-aminosalicylates (5-ASA), steroids, azathioprine or methotrexate, and infliximab. Mucosal healing has been assessed in recent trials with different formulations of 5-ASA. In the ASCEND studies, evaluating different dosages of a delayed-released oral mesalazine in patients with mild or moderate UC, complete remission (including endoscopic remission) ranged between 18% and 25% at

week 6[35,36]. Truelove *et al*[37] demonstrated in 1954 that mucosal healing can be obtained with a high-dose of oral steroids in 30% of patients at week 6 compared with 10% in patients who received placebo ($P = 0.02$). In a recent review, it was considered that corticosteroids induce mucosal healing in 12%-41% of patients with UC, depending on the method of administration and the medication[10]. Some data suggest that mucosal healing may also be obtained with azathioprine or methotrexate[38,39]. Anti-TNF agents probably induce mucosal healing more rapidly. In ACT 1 and ACT 2, patients with refractory moderate-to-severe UC received placebo or infliximab intravenously[40]. Induction therapy with infliximab resulted in mucosal healing at week 8 in 61% of patients (148/242) compared with 32% (79/244) in the placebo groups ($P < 0.001$)[40]. At week 54 (ACT 1), scheduled maintenance therapy with infliximab resulted in mucosal healing in 45.5% (55/121) of patients compared with 18.2% (22/121) in the placebo group ($P < 0.001$).

Data from several studies suggest that mucosal healing may be associated with a better outcome in UC, more specifically a decreased risk of relapse. Reduced relapse rates have been demonstrated in UC patients who achieved mucosal healing with steroids. In a study published in 1966, Wright *et al*[41] found that 40% of patients who achieved mucosal healing with oral and rectal steroids did not relapse during 1 year of follow up as compared to 18% of those who still had lesions. In the ACT1 and 2 studies on infliximab maintenance in patients with moderately to severely active UC, 48.3% of the patients who achieved mucosal healing at week 8 were in remission at week 30 as compared to only 9.5% of those who did not achieve mucosal healing[39].

Mucosal healing may also be associated with reduced risk of surgery in UC. In the IBSEN population-based study, UC patients who achieved mucosal healing at 1 year (whatever the treatment) had a decreased risk of colectomy at 5 years (2% *vs* 7%, $P = 0.02$)[16]. A study performed in the Leuven cohort of UC patients treated with infliximab showed that colectomy was more frequent in patients who did not achieve mucosal healing at week 4 or 10 (Mayo endoscopic subscore greater than 1)[42]. In ACT1 and 2, it was shown that patients treated with infliximab were less likely to undergo colectomy through 54 wk than those receiving placebo[43]. However, data on the relationship between mucosal healing and risk of colectomy are not available in these studies.

Finally, there is a clear relationship between the grade and chronicity of inflammation in the colon and the risk of colorectal cancer. Better control of inflammation, as demonstrated with mucosal healing, may be associated with decreased risk of colorectal cancer.

PERSPECTIVES

There is growing evidence that morphological evaluation of lesions may provide help in the selection of IBD patients who should be treated more intensively. Endoscopy may help to select patients who will better respond to a therapy and those who should be treated because of exposure to an increased risk of complication and surgery. Assessment of mucosal healing should be increasingly performed in the future. However, it is not yet proven that patients not achieving mucosal healing should be treated more intensively. As endoscopy may be difficult to repeat in these patients, surrogate markers such as calprotectin or C-reactive protein levels, provided they are closely related to the presence of severe lesions, could be increasingly used.

Another limit of endoscopy relies on the fact that only mucosal aspects can be assessed by endoscopy. Radiological techniques, including ultrasonography, CT scan or MRI, can also describe the wall and identify penetrating and stricturing complications. Recent studies with MRI suggest that this technique could be proposed to assess severity of mucosal and parietal disease[44].

CONCLUSION

Endoscopy, which allows a direct assessment of severity and extent of mucosal lesions, may help in the management of IBD. In patients with active IBD, endoscopy may help to select patients who should receive early and active therapies and this is for two reasons. First, severe endoscopic lesions may predict a poor outcome with increased risk of colectomy and complications. Second, patients with no lesions gain no benefit in receiving active treatments with potential risks. In treated IBD patients, mucosal healing is associated with a better outcome, with decreased risks of relapse and major surgery. Assessment of mucosal healing may help to characterize the response to treatments and in decisions of optimal strategies.

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REVIEW

Heme oxygenase-1 system and gastrointestinal tumors

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Abstract

Heme oxygenase-1 (HO-1) system catabolizes heme into three products: carbon monoxide, biliverdin/bilirubin and free iron. It is involved in many physiological and pathophysiological processes. A great deal of data has demonstrated the roles of HO-1 in the formation, growth and metastasis of tumors. The interest in this system by investigators involved in gastrointestinal tumors is fairly recent, and few papers on HO-1 have touched upon this subject. This review focuses on the current understanding of the physiological significance of HO-1 induction and its possible roles in the gastrointestinal tumors studied to date. The implications for possible therapeutic manipulation of HO-1 in gastrointestinal tumors are also discussed.

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Key words: Heme oxygenase-1; Gastrointestinal tumors

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INTRODUCTION

Heme oxygenase (HO) plays an important role in regulating the intracellular heme level by cleaving heme into carbon monoxide (CO), biliverdin and free iron[1]. Three HO isoforms have been identified to date: HO-1, HO-2, and HO-3, among which the isoforms 1 and 2 are the best known. HO-2 is constitutively and most highly expressed in neuronal tissues contributing to cell homeostasis, whereas HO-1, also referred to as heat shock protein-32 (Hsp32), is an inducible enzyme and relatively lowly expressed in most tissues[2]. HO-3, which is described in the rat brain, has no activity and is not expressed in humans[3].

HO-1 and HO-2 are different gene products. Unlike the constitutively expressed HO-2, HO-1 is exquisitely sensitive, not only to heavy metals[4], but to all kinds of stimuli and agents that cause oxidative stress and pathological conditions, including ischemia[5], hemorrhagic shock[6], heat shock[7], hypoxia[8], UVA radiation[9], reactive oxygen species (ROS)[10]. In fact, there is no other enzyme to date that is affected by so many stimuli of diverse nature as HO-1[2].

HO-1 is involved in many pathophysiological processes, ranging from Alzheimer's disease to cancer. The interest in this system by investigators involved in gas-

trointestinal tumors is fairly recent, and few reports on HO-1 have focused on this subject.

HO-DERIVANTS AND THEIR EFFECTS

All products of HO activity are now suspected to be biologically active, in which metabolic pathway is involved in a wide variety of physiological and pathophysiological processes[11]. Almost all CO produced *in vivo* comes from the degradation of heme by HO. Depending on the cell type, CO can activate one or both key signaling pathways in numerous physiological and pathophysiological conditions (Figure 1). One of the pathways is soluble guanylate cyclase (sGC)/cyclic guanosine monophosphate (cGMP), which has been implicated in mediating the effects of CO on vascular contractility, the inhibition of smooth muscle proliferation, neurotransmission[12,13] and preventing apoptosis in endothelial cells[14] and fibroblasts[15]. Another one is p38 mitogen-activated protein kinase (MAPK) pathway, through which CO can mediate the anti-inflammatory actions in a large measure[16-18]. Moreover, Chin *et al*[19] recently pointed out that CO has played an additional novel role as a host defense molecule agent against microbes (bactericidal agent).

HO-1 catalyzes the rate-limiting step in heme degradation to biliverdin. Biliverdin is, in turn, converted into bilirubin by biliverdin reductase at the expense of NADPH. Biliverdin and bilirubin are potent antioxidants[20,21]. Several studies have demonstrated that the administration of biliverdin and/or bilirubin is potently cytoprotective in a variety of pathophysiological events, including ischemia-reperfusion injury, transplant rejection and inflammatory bowel disease[22-25]. In addition, bilirubin is also known to modulate immune effector functions and suppress inflammatory response[26].

Fe^{2+}, which is also a product of heme degradation, upregulates an iron-transporter pump that removes intracellular Fe^{2+} from the cell[27] and induces the expression of ferritin, a iron binding protein[28]. Expression of ferritin is originally reported to protect endothelial cell against oxidant damage *in vitro*[28]. In addition, over-expression of H-ferritin (heavy chain ferritin) has also been shown to protect cultured endothelial cells from undergoing apoptosis and protect livers from transplant-associated ischemia-reperfusion injury[29]. Although the roles of the iron and ferritin in the overall cytoprotective effect of HO-1 are not clear, presumably both contribute in a crucial manner to the overall anti-oxidant effect following increased HO-1 expression in a variety of situations[30].

EXPRESSION OF HO-1 IN GASTROINTESTINAL TUMORS

Expression of HO-1 is usually increased in tumors, compared with surrounding healthy tissues, which was shown in oral squamous cell carcinoma[31], pancreatic cancer[32] and hepatoma[33]. There have been few published reports in gastrointestinal tumors and only one paper to date has investigated HO-1 expression in gastrointestinal tumors.

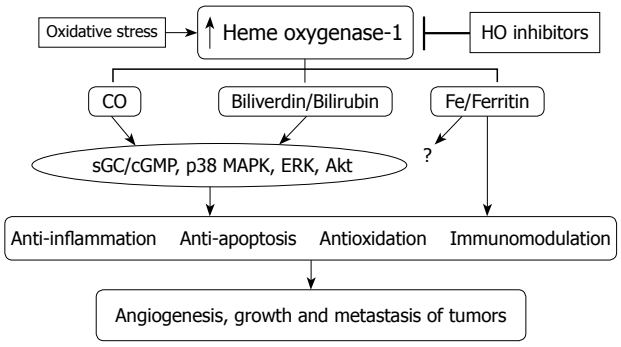


Figure 1 Schematic demonstration of mechanism underlying the biological actions of HO-1 pathway in tumors. HO-1: Heme oxygenase-1; CO: Carbon monoxide; sGC/cGMP: Soluble guanylate cyclase/cyclic guanosine monophosphate; MAPK: Mitogen-activated protein kinase.

The study investigates the expression of HO-1 in human colon tumor. Focal HO-1 expression in colorectal cancer and colonic adenoma was found to be 41.8% (23/55) and 36.8% (7/19), respectively[34]. Of importance, HO-1 in gastrointestinal tumors can be further upregulated in response to chemotherapy[35] and photodynamic therapy[36].

HO-1 PROMOTER POLYMORPHISM

HO-1 is known as an oxidative stress responsive protein that is upregulated by multiple stimuli, which has been proposed to provide an important cellular response that protects cells against oxidative damage. However, humans differ quantitatively in their ability to mount an HO-1 response. The association between the HO-1 genotype and various gastrointestinal tumors has been studied. Lo *et al*[37] examined the correlation between the HO-1 gene promoter polymorphism and the clinicopathological characteristics, along with the risk of gastric cancer. Their findings suggest that the long (GT)n repeat of HO-1 gene promoter was associated with a higher frequency of gastric adenocarcinoma, and the medium (GT)n repeat might possess a protective effect against gastric adenocarcinoma with a lower frequency of lymphovascular invasion in tumors. Additionally, the (GT)n-repeat promoter polymorphism was investigated in gastrointestinal stromal tumors (GIST) by Vashist *et al*[38]. They found that Short GTn allele (SGTn) was significantly associated with metastasis, higher tumor recurrence rates and high-risk GIST. Furthermore, SGTn allele carriers had significantly shorter disease-free and overall survival[38]. Moreover, a higher promoter activity genotype of the HO-1 gene was associated with increased risk in women with gastric cancer[39]. In gastrointestinal tumors, a potential impact of the (GT)n repeat polymorphism has been demonstrated, which might be considered as a potential prognostic marker to allocate patients to different risk groups and customize therapy and follow-up.

HO-1 AND CYTOPROTECTION AND APOPTOSIS

The gastrointestinal tract is lined by a simple epithelium

that undergoes constant renewal involving cell division, differentiation and cell death, in which HO-1 plays a major role in the regulation of the cell cycle/apoptosis, oxidative stress, inflammation, development of colon cancer[40]. Many studies have convincingly shown that HO-1 is a cytoprotective and antiapoptotic enzyme in gastrointestinal tumor cells exposed to diverse stimuli (Figure 1). It has been demonstrated that HO-1 is involved in the pathogenesis of human gastric cancer. Antiapoptotic effects of HO-1 in gastric cancer cells are independent of p53 status in a p38 MAPK- and ERK-mediated pathway with elevated caspase inhibitory protein-2 (c-IAP2) and decreased caspase-3 activity[41], in which nuclear factor-κB is implicated. The pathway of HO-1 was also investigated in HT29 human colon cancer cells by Park *et al*[42]. Another study also demonstrated that HO-1 induction resulted in resistance to apoptosis in a human colon cancer cell line, Caco-2, whose effects were independent of p38, but were mediated *via* Akt pathway[43]. In a similar study, Kim *et al*[44] reported that administration of Zerumbone (ZER) effectively suppressed mouse colon carcinogenesis through multiple modulation of growth, apoptosis and inflammation. Ohyama *et al*[45] examined the cytotoxicity of a crude extract from Vitex agnus-castus fruits (Vitex extract) in gastric signet ring carcinoma (KATO-III) cells. They found that cell apoptosis may be attributed to the inhibition of HO-1. It can be supposed that cytoprotective action of HO-1 can be mediated by the following factors: (a) decreased intracellular pro-oxidant levels; (b) increased bilirubin levels; and (c) elevated CO production[46]. On the contrary, flavonoids- (Vitex extract) induced apoptosis is caused through the induction of HO-1 in human colon carcinoma cell line, COLO 201[35]. The relationship between HO-1 and apoptosis remains to be clarified.

HO-1 AND TUMOR GROWTH AND METASTASIS

Apart from the cytoprotective action, HO-1 is commonly regarded as a potent proangiogenic enzyme. Angiogenesis is critical not only for tumor growth but also for metastasis. Thus, proangiogenic action of HO-1 may further support tumor progression[47]. Bussolati *et al*[48] reported that vascular endothelial growth factor (VEGF) induced prolonged HO-1 expression and activity in human endothelial cells and HO-1 inhibition abrogated VEGF-driven angiogenesis. Overexpression of HO-1 in pancreatic cancer cells[49] and melanoma cells[50] increased the occurrence of metastasis, while inhibition of HO activity completely inhibited the occurrence of metastasis[49]. In contrast, some authors have demonstrated that inhibition of the HO pathway by zinc deuteroporphyrin 2,4-bis glycol (ZnDPBG) in colon carcinoma had no effect on metastasis to the lung and even increases metastasis to the liver[51]. Furthermore, the rate of lymphatic tumor invasion was significantly lower in colorectal cancer samples expressing HO-1[34]. Thus, the mechanism of HO-1 in the metastatic potential of cancer cells is not recognized and it may depend on the type of cancer or other still not defined factors.

HO-1 AS A POTENTIAL THERAPEUTIC TARGET

Studies of the role of HO-1 seem to be important not only for better understanding of tumor growth regulation but also for clinical practice. HO-1 is often upregulated in gastrointestinal tumors[34], its expression is further increased in response to the different types of therapies such as photodynamic therapy[52,53] and pyrrolidine dithiocarbamate[54]. Since HO-1 may protect tumor cells against oxidative stress and can be regarded as an enzyme facilitating tumor progression, administration of HO-1 inhibitors might be effective for the treatment of gastrointestinal tumors. It has been demonstrated that pegylated zinc protoporphyrin, a potent HO inhibitor, administered *in vitro* induced apoptosis of human colon carcinoma SW480 cells and inhibited growth of murine colon carcinoma *in vivo*[55]. However, the growth, invasion and metastasis of tumors are a highly complex and multistep process, the mechanisms responsible for HO-1 in gastrointestinal tumors remain to be elucidated.

CONCLUSION

HO-1 system may play an important role in different pathophysiological conditions, in which pharmacologic modulation of HO-1 system may represent an effective and cooperative strategy to facilitate tumor growth and metastasis, although the exact effects can depend on the type of disease. Therefore, down-regulating the HO-1 system by pharmacological or genetic means will be a new therapeutic approach in the management of gastrointestinal tumors. A comprehensive understanding of the underlying mechanisms for the observed effects of HO-1 and its products will be necessary before their use can be evaluated in clinical applications for the prevention and/or treatment of human diseases such as gastrointestinal tumors.

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ORIGINAL ARTICLE

Antioxidant and immunomodulatory effects of Viusid in patients with chronic hepatitis C

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Abstract

AIM: To investigate the efficacy of Viusid, a nutritional supplement, as an antioxidant and an immunomodulator in patients with chronic hepatitis C.

METHODS: Sixty patients with chronic hepatitis C who were non-responders to standard antiviral treatment were randomly assigned to receive Viusid (3 sachets daily, n = 30) or placebo (n = 30) for 24 wk. The primary outcome was the change in serum malondialdehyde and 4-hydroxyalkenals (lipid peroxidation products). Secondary outcomes were changes in serum tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and interleukin-10 (IL-10).

RESULTS: Statistically significant reductions in serum 4-hydroxyalkenals and malondialdehyde levels were observed in both groups in comparison with pretreatment values, but the patients who received Viusid showed a more marked reduction as compared with the control group (P = 0.001). TNF-α levels significantly increased from 6.9 to 16.2 pg/mL (P < 0.01) in the patients who received placebo in comparison with almost unchanged levels, from 6.6 to 7.1 pg/mL (P = 0.26), in the patients treated with Viusid (P = 0.001). In addition, IL-10 levels were markedly increased in the patients treated with Viusid (from 2.6 to 8.3 pg/mL, P = 0.04) in contrast to the patients assigned to placebo (from 2.8 to 4.1 pg/mL, P = 0.09) (P = 0.01). Likewise, the administration of Viusid markedly increased mean IFN-γ levels from 1.92 to 2.89 pg/mL (P < 0.001) in comparison with a reduction in mean levels from 1.80 to 1.68 pg/mL (P = 0.70) in the placebo group (P < 0.0001). Viusid administration was well tolerated.

CONCLUSION: Our results indicate that treatment with Viusid leads to a notable improvement of oxidative stress and immunological parameters in patients with chronic hepatitis C.

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Key words: Antioxidant therapy; Chronic hepatitis C; Cytokines; Immunomodulatory therapy; Nutritional supplement; Oxidative stress

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INTRODUCTION

Hepatitis C virus (HCV) infection is one of the most important causes of chronic liver disease leading to cirrhosis and hepatocellular carcinoma (HCC)[1]. Peginterferon plus ribavirin is the only therapeutic alternative with proven efficacy in patients with chronic hepatitis C (CHC)[2]. Unfortunately, sustained virological response is achieved in only approximately 50% of the patients. HCV protease and polymerase inhibitors have been evaluated recently, but the increased rate of adverse events and discontinuations reported during the trials may possibly preclude their use in clinical practice[3,4].

The exact pathogenesis of liver injury and fibrosis has not been fully elucidated. Previous studies have found increased oxidative damage in patients chronically infected with HCV[5,6]. These findings have been supported by increased levels of lipid peroxidation (LPO) products (such as malondialdehyde and hydroxynonenal), an overabundance of reactive oxygen species, elevated superoxide dismutase (SOD) activity and reduced levels of glutathione (GSH) in serum or erythrocytes of patients with CHC compared with healthy controls[5-9].

Similarly, cytokine dysregulation is thought to play a crucial role in the persistence of viral infection and as a key mediator in the inflammatory and fibrogenic processes of patients with HCV infection[10].

Studies have shown that increased production of interferon-γ (IFN-γ) is strongly associated with spontaneous resolution of HCV infection compared with scant production in patients with persistent infection[11,12]. Likewise, it has been reported that patients with chronic HCV infection show enhanced serum interleukin (IL)-10 concentration[13,14]; however, long-term IL-10 therapy to treat chronically HCV-infected patients leads to a significant improvement of inflammation and fibrosis[15].

Several studies have suggested a positive association between increased serum tumor necrosis factor-α (TNF-α), IL-1 and -6 levels, and the degree of hepatic inflammation and fibrosis in patients with CHC[16,17].

This provides a rationale for studies using antioxidant and immunomodulatory agents; however, trials involving these agents have not clearly demonstrated their potential benefits, in part due to the limitations of non-randomized studies with small sample size, lack of dependable end points, and poor understanding of the role of different antioxidants in CHC[18-23].

There is an obvious need for the continuous development of new treatment strategies for CHC. Thus, the administration of compounds with antioxidant and immunomodulatory properties could be a plausible strategy to halt the natural course of the disease, particularly in patients with poor response to antiviral therapy. Viusid, a nutritional supplement, contains different molecules (ascorbic acid, zinc, and glycyrrhizic acid) with recognized antioxidant properties (Table 1)[24-26]. Its different chemical compounds are activated through a molecular activation principle that strongly increases their biological activity without modifying their physical structure. Recently, Vilar Gomez *et al*[27] reported that the addition of Viusid to the conventional interferon/ribavirin therapy was associated with significant histologic and biochemical improvements, especially in patients without sustained virological response. They suggested that the Viusid-related effect on histologic features, especially fibrosis, appears to be associated with antioxidant and/or immunomodulatory properties. All of these effects could modulate the histologic pattern of CHC, especially inflammation and fibrosis, in an attempt to halt disease progression. Therefore, we conducted a randomized double-blind and placebo-controlled study to evaluate whether Viusid may have a beneficial effect on oxidative stress and cytokine parameters in patients with chronic hepatitis C.

MATERIALS AND METHODS

Patients

We recruited 60 patients with chronic hepatitis C diagnosis at a tertiary care center (National Institute of Gastroenterology, Havana, Cuba).

Inclusion criteria included male and female patients of 18 to 65 years of age who had a positive test for anti-HCV antibody, HCV RNA detectable in serum by polymerase chain reaction (PCR), persistently elevated ALT at least on two occasions, liver biopsy consistent with chronic hepatitis, non-responders to previous treatment with peginterferon plus ribavirin, and absence of significant alcohol ingestion (weekly ethanol consumption of more than 40 g).

Exclusion criteria included presence of any other form of liver disease, positive screening for viral hepatitis A and B and human immunodeficiency virus (HIV), pregnancy or lactation, decompensated cirrhosis, concomitant disease with reduced life expectancy, severe psychiatric conditions, drug dependence, evidence of liver cancer at entry into the study on the basis of ultrasonography and α-fetoprotein levels less than 50 ng/L, and refusal to participate in the study.

Ethics

The study was conducted in compliance with the Declaration of Helsinki and approved by the ethics committee and the institutional review board of the National Institute of Gastroenterology. All patients provided written

Table 1 Ingredients of Viusid

Malic acid	0.666 g	Ascorbic acid	0.020 g
Glycyrrhizic acid	0.033 g	Folic acid	66 mcg
Glucosamine	0.666 g	Cyanocobalamine	0.3 mcg
Arginine	0.666 g	Zinc sulfate	0.005 g
Glycine	0.333 g	Pyridoxal	0.6 mg
Calcium pantothenate	0.002 g		

informed consent for participation. The trial had been registered at ClinicalTrials.gov (NCT00778843).

Study design

After initial evaluation, all patients who met the eligibility criteria were consecutively enrolled in the study. They were randomly assigned to receive: Viusid (3 sachets daily, $n = 30$) or placebo (3 sachets daily, $n = 30$) for 24 wk. Viusid was provided by Catalysis, S.L. (Madrid, Spain).

Randomization was conducted by allocation into blocks of 4 (block randomization). It was performed by a health worker experienced in randomization techniques who was not involved in the evaluation or treatment of the participants. The physicians, study coordinators, and patients did not have access to the randomization scheme.

The patients, investigators, and study coordinators were blinded as to the treatment administered. Catalysis, Spain provided Viusid and placebo sachets. There was no difference in appearance, smell or flavor between Viusid and placebo.

All patients were evaluated at baseline and at monthly intervals by an experienced hepatologist who strongly encouraged the subjects to accomplish the treatment.

Clinical assessment, including body weight, liver tests, glucose, creatinine, and hemoglobin determinations, along with compliance with the study medication (verified through sachet count) and adverse events, was determined at baseline and/or at monthly intervals during the 24 wk of the study.

The HCV-RNA level was quantified by PCR assay (Amplicor Monitor HCV v. 2.0; Roche Molecular Systems; lower limit of detection, 600 IU/mL). HCV genotyping was performed by reverse hybridization (Inno-LiPA HCV; Innogenetics, Ghent, Belgium). Liver biopsy was performed at least 6 mo before the inclusion in the study and histological results were classified by a single local pathologist according to Ishak score. Liver biopsies were also performed after conclusion of the antiviral treatment.

Laboratory method

Venous blood samples were collected after an overnight fast; 7 mL of blood was drawn into tubes containing EDTA. Plasma was obtained by centrifugation (4000 *g* for 15 min at 4℃) and frozen at -70℃ until assayed. After separation of the plasma, the buffy coat was removed and the packed cells washed three times with cold isotonic saline. Then, a known volume of erythrocytes was lysed with cold distilled water (1:4) and stored in a refrigerator at 4℃ for 15 min, and the cell debris was removed by centrifugation. The erythrocyte lysates were stored at -70℃ until assayed. In general, all samples were analyzed in duplicate.

Oxidative stress determination: Blood for oxidative stress determination was drawn at baseline and every two months up to study conclusion.

The blood oxidative stress status was evaluated by assaying nine parameters: plasma lipid peroxidation products (malondialdehyde and 4-hydroxyalkenals), advanced oxidation protein products, and antioxidant defense systems (glutathione peroxidase, Cu/Zn superoxide dismutase, catalase, glutathione reductase, glutathione, and myeloperoxidase).

Erythrocyte catalase, glutathione reductase, and glutathione peroxidase activities were determined with BIOXYTECH® CAT-520™, BIOXYTECH® GR-340™ and BIOXYTECH® GPx-340™ (OXIS Research, Portland, USA), respectively. Their values were expressed as units per milligram of hemoglobin.

SOD in erythrocytes was measured by the Fulbert method[28], based on the capacity of SOD to inhibit the reduction of ferricytochrome C by the xanthine/xanthine oxidase system. A standard curve was prepared using commercially available SOD (Sigma-Aldrich). Reaction development was monitored spectrophotometrically at 405 nm. One SOD unit is defined as the amount of enzyme that inhibits cytochrome C reduction by 50% under the respective conditions; activity is expressed per hemoglobin.

LPO measurement was performed using the BIOXYTECH® LPO-586™ kit (OXIS Research, Portland, USA) and was based on the reaction of a chromogenic reagent (N-methyl-2-phenylindole) with malondialdehyde (MDA) and 4-hydroxyalkenals at 45℃.

Determination of advanced oxidation protein products (AOPP) was based on spectrophotometric detection according to the method described by Witko-Sarsat[29]. Briefly, 200 μL of plasma [diluted 1:5 with phosphate buffered saline (PBS)], 200 μL of chloramine-T (0-100 μmol/L) for calibration, and 200 μL of PBS as blank were applied on a microtiter plate. 10 μL of 1.16 mol/L potassium iodide and 20 μL of acetic acid were added to each well, and absorbance at 340 nm was measured immediately. Concentration of AOPP was expressed in chloramine units (μmol/L).

GSH concentration was measured by the method of Sedlak and Lindsay, in which 5,5'-dithiobis-(2-nitrobenzoic acid) reacting with sulfhydryl groups yields 2-nitro-5-mercaptobenzoic acid with maximal absorbance at 412 nm[30]. Glutathione (Sigma-Aldrich) was used to generate the standard curves.

Serum myeloperoxidase (MPO) concentration was measured using a high-sensitivity, quantitative sandwich enzyme immunoassay (OXIS Research, Portland, USA). In this assay, the lowest detection limit of MPO is 1.02 ng/mL.

Serum cytokine determinations: Blood for cytokine measurement was drawn at baseline and every two months up to study conclusion.

Serum cytokine levels (IL-1α, IL-1β, IL-2, IL-6, IL-10,

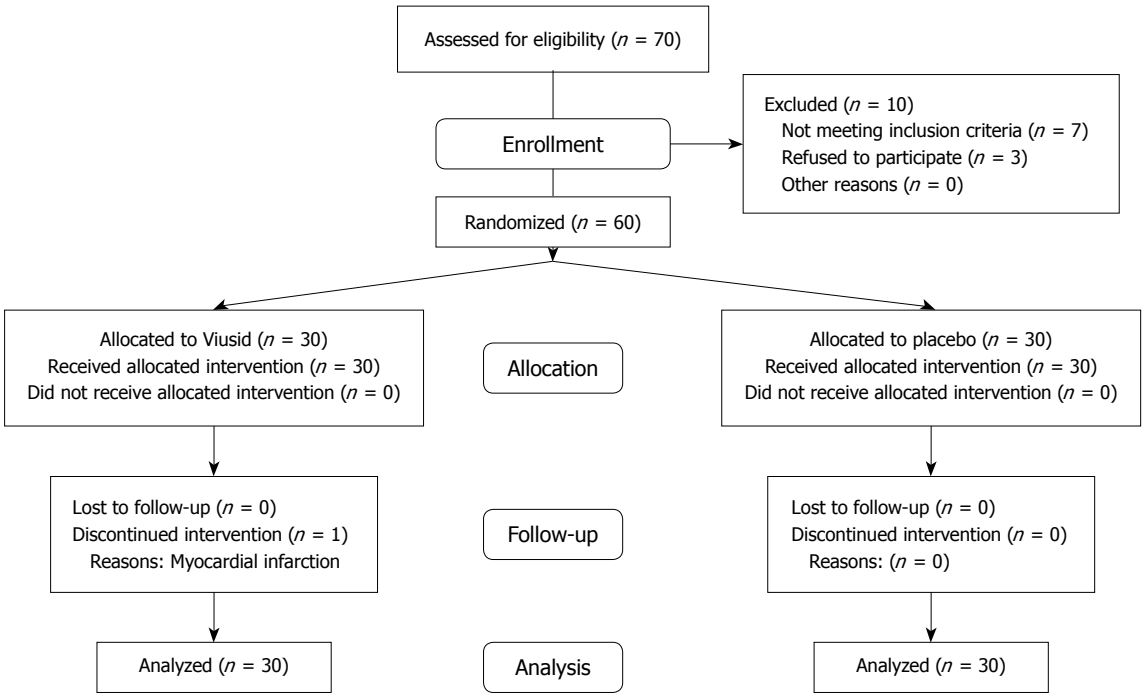


Figure 1 Flow of participants through the study.

IL-12; TNF-α, TNF-β, IFN-γ, cathepsin L, and GM-CSF) were measured using commercial sandwich-type ELISA kits following the procedure recommended by the manufacturers (Bender MedSystem, Vienna, Austria). Detection limits were as follows: 1.1 pg/mL for IL-1α, 0.3 pg/mL for IL-1β, 9.9 pg/mL for IL-2, 0.92 pg/mL for IL-6, 1.0 pg/mL for IL-10, 2.1 pg/mL for IL-12; 2.3 pg/mL for TNF-α, 4.6 pg/mL for TNF-β, 0.99 pg/mL for IFN-γ, 1.71 ng/mL for cathepsin L, and 2.9 pg/mL for GM-CSF.

Outcomes

The primary outcome was the change in serum MDA and 4-hydroxyalkenals (lipid peroxidation products). Secondary outcomes were changes in serum TNF-α, IL-10 and IFN-γ. Change was the difference between measurements obtained at baseline and at the end of treatment (24 wk).

Statistical analysis

The baseline characteristics were summarized as percentages for categorical variables and as mean ± SE for continuous variables. The χ^2 test was applied to categorical variables. The two-sample *t*-test was used to compare means, and the Mann-Whitney *U*-test if they were not normally distributed. Outcome measurements included all patients who were randomized and received at least one dose of study medication (intention-to-treat analysis). The Wilcoxon signed-rank tests were used to compare changes between the baseline and post-treatment measurements, and the Wilcoxon rank-sum tests were used for treatment group comparisons. The safety analysis included all treated patients who had at least one safety evaluation after baseline.

The study was designed to have a statistical power of 80% to detect an absolute difference of 28% in the changes of serum MDA and 4-hydroxyalkenals (28% in the group with Viusid versus 0% in the placebo group). Considering a type I error of 0.05 and a type II error of 0.20, 30 patients per arm were needed to reach statistical significance.

All confidence intervals, significance tests, and resulting *P* values were two-sided, with an alpha level of 0.05.

Statistical analyses were performed using SPSS Inc. for Windows, release 15, Chicago, IL.

RESULTS

A total of 60 patients were recruited at a tertiary center (National Institute of Gastroenterology, Havana, Cuba) between October 2008 and May 2009. The flow of participants through the trial is presented in Figure 1. All randomized patients received at least one dose of the study medication; 1 subject assigned to Viusid did not complete the study and the reason for discontinuation was a myocardial infarction in week 16 of the study. No patient was lost during the follow-up period and no patient received co-interventions during the trial that could have affected the outcomes.

All patients who underwent random allocation were analyzed for outcomes according to original group assignment (intention-to-treat analysis).

Baseline characteristics were comparable across the two groups (Table 2). The majority of the patients in the Viusid group were female; however, there was no statistical difference in comparison with the placebo group. All patients were non-responders to previous antiviral therapy with peginterferon plus ribavirin and infected with genotype 1, and more than half of the patients had a viral load of over 600 000 IU/mL (75%).

Table 2 Baseline characteristics

Variable	Viusid (n = 30)	Placebo (n = 30)	P value[1]
Age (yr)	49.3 ± 2.1	49.7 ± 2.2	0.90
Sex, n (%)			
Male	10 (33)	16 (53)	0.19
Female	20 (67)	14 (47)	
Body mass index (kg/m^2)	25.3 ± 0.7	26.4 ± 0.7	0.28
HCV RNA > 600000 IU/mL, n (%)	24 (80)	21 (70)	0.36
Genotype 1, n (%)	30 (100)	30 (100)	1.00
Ishak inflammation score	5.98 ± 3	5.88 ± 3	0.55
Ishak fibrosis score	1.88 ± 0.91	2.06 ± 1.8	0.68
ALT (IU/L)	66.9 ± 7.6	75.4 ± 8.4	0.45
AST (IU/L)	55.9 ± 7	65.8 ± 7.6	0.34
Fasting plasma glucose (mmol/L)	5.1 ± 0.14	5.8 ± 0.4	0.09
Alkaline phosphatase (mmol/L)	217.8 ± 12.8	221.1 ± 13.7	0.86
Creatinine (mmol/L)	80.4 ± 2.8	82.8 ± 3.6	0.59
Hemoglobin (g/L)	126.4 ± 1.4	131.3 ± 2.7	0.11
γ-glutamyltransferase (IU/L)	72.7 ± 13.5	84.1 ± 13.8	0.55
Bilirubin (mmol/L)	11.4 ± 1.4	14.1 ± 1.3	0.16
Albumin (g/L)	47.8 ± 0.5	47.6 ± 0.9	0.80
Uric acid (mmol/L)	257 ± 16.8	276 ± 13.4	0.37
IL-1α (pg/mL)	3.26 ± 0.7	3.09 ± 0.5	0.89
IL-1β (pg/mL)	3.7 ± 0.1	4 ± 0.1	0.29
IL-2 (pg/mL)	17.9 ± 2.3	19.9 ± 4.7	0.93
IL-6 (pg/mL)	2.5 ± 0.2	2.1 ± 0.3	0.35
IL-10 (pg/mL)	2.6 ± 0.6	2.8 ± 4.1	0.67
IL-12 (pg/mL)	7.5 ± 1.4	7.6 ± 1.2	0.92
IFN-γ (pg/mL)	1.92 ± 0.2	1.80 ± 0.1	0.63
TNF-α (pg/mL)	6.6 ± 0.2	6.9 ± 0.5	0.45
TNF-β (pg/mL)	8.5 ± 0.4	9.1 ± 0.6	0.58
Cathepsin L (ng/mL)	1.7 ± 0.1	1.7 ± 0.1	0.99
GM-CSF (pg/mL)	4.4 ± 0.1	4.3 ± 0.1	0.97
MDA (μmol/L)	0.97 ± 0.05	1.12 ± 0.09	0.17
4-hydroxyalkenals (μmol/L)	1.75 ± 0.09	1.92 ± 0.14	0.30
Cu/Zn SOD (U/mg Hb)	17.6 ± 1.1	18.7 ± 0.9	0.46
CAT (U/mg Hb)	235 ± 23.7	289 ± 21.8	0.09
GR (mU/mg Hb)	6.1 ± 0.7	6.9 ± 1.1	0.73
GPx (mU/mg Hb)	169 ± 11.6	176 ± 11.2	0.66
GSH (μmol/L)	26.3 ± 2.3	31.8 ± 3.2	0.16
MPO (ng/mL)	221 ± 6.6	221 ± 6.7	0.91
AOPP (μmol/L)	36.1 ± 5.4	26.2 ± 5.5	0.20

[1]P values are for the comparison between Viusid and placebo; Plus-minus values are mean ± SE of mean; For all laboratory measures and for continuous demographics: P value Mann-Whitney U-test; Proportions: Percentage, P value χ^2. HCV: Hepatitis C virus; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; IL: Interleukin; IFN: Interferon; TNF: Tumor necrosis factor; GM-CSF: Granulocyte-macrophage colony-stimulating factor; MDA: Malondialdehyde; Cu/Zn SOD: Copper/Zinc superoxide dismutase; CAT: Catalase; GR: Glutathione reductase; GPx: Glutathione peroxidase; GSH: Glutathione; MPO: Myeloperoxidase; AOPP: Advanced oxidation protein products.

Oxidative stress markers

Lipid peroxidation products (primary end point): There were significant mean reductions from baseline to 6 mo in MDA and 4-hydroxyalkenals in each group of treatment (Table 3). However, the administration of Viusid markedly reduced the mean MDA level from 0.97 to 0.29 μmol/L with a mean improvement of 0.68 (95% CI: 0.50-0.84) *vs* 1.12 to 0.76 μmol/L with a mean improvement of 0.36 (95% CI: 0.11-0.62) for placebo (P = 0.001) in comparison with baseline values (Figure 2A). Similarly, significant changes in the mean of serum 4-hydroxyalkenals occurred in the patients assigned to Viusid (1.76 to 0.53 μmol/L), with a mean difference of 1.22 (95% CI: 0.97-1.45), as compared with the patients treated with placebo (1.92 to 1.39 μmol/L), with a mean difference of 0.53 (95% CI: 0.43-1.17) (Figure 2B).

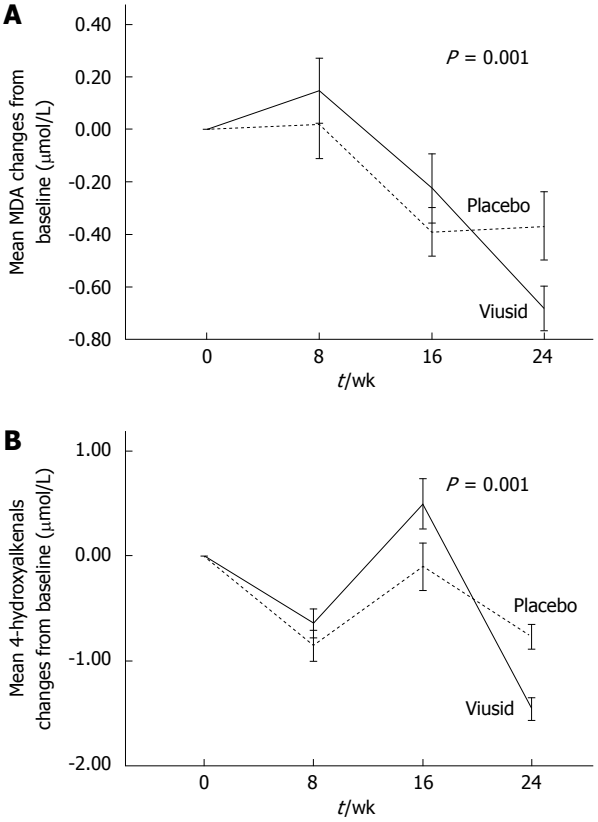


Figure 2 Change in mean serum malondialdehyde (MDA) (A) and serum 4-hydroxyalkenals (B) levels during the treatment period. Error bars indicate SE. P value for the comparison of the Viusid and the control group at 24 wk. A comparative analysis was performed using Wilcoxon rank-sum test.

Antioxidant defense system markers: Surprisingly, significant reductions in erythrocyte Cu/Zn SOD, CAT, GR, and serum GSH and MPO were observed in both groups of treatment in comparison with pretreatment values; however, no significant difference was observed between groups (Table 3).

Advanced oxidation protein products: Serum AOPP concentrations remained unchanged for the patients who received Viusid or placebo as compared with baseline values (Table 3).

Serum cytokines

Serum cytokine results are summarized in Table 4.

TNF-α levels (secondary outcome) significantly increased from 6.9 to 16.2 pg/mL (P < 0.01) in the patients treated with placebo in comparison with almost unchanged levels (6.6 to 7.1 pg/mL, P = 0.26) in the patients treated with Viusid (P = 0.001) (Figure 3A). Similarly, the administration of Viusid markedly increased the mean IFN-γ level (secondary outcome) from 1.92 to 2.89 pg/mL (P < 0.001) in comparison with re-

Table 3 Patients' baseline oxidative stress parameters and outcome at 6 mo

Variable	Viusid (n = 30)				Placebo (n = 30)				P value[1]
	Before treatment	After treatment	Change	P value	Before treatment	After treatment	Change	P value	
MDA (μmol/L)	0.97 ± 0.05	0.29 ± 0.05	-0.68 ± 0.08	< 0.001	1.12 ± 0.09	0.76 ± 0.08	-0.36 ± 0.12	0.002	0.001
4-hydroxyalkenals (μmol/L)	1.75 ± 0.09	0.53 ± 0.06	-1.22 ± 0.1	< 0.001	1.92 ± 0.14	1.39 ± 0.13	-0.53 ± 0.2	0.01	0.001
Cu/Zn SOD (U/mg Hb)	17.6 ± 1.1	3.26 ± 0.4	-14.3 ± 1.2	< 0.001	18.7 ± 0.9	3.44 ± 0.39	-15.3 ± 1.04	< 0.001	0.71
CAT (U/mg Hb)	235 ± 23.7	17.6 ± 2.3	-217.4 ± 24	< 0.001	289 ± 21.8	21 ± 2.7	-268 ± 22	< 0.001	0.13
GR (mU/mg Hb)	6.1 ± 0.7	11 ± 1.1	4.9 ± 1.3	< 0.001	6.9 ± 1.1	12 ± 1.2	5.1 ± 1.6	0.001	0.97
GPx (mU/mg Hb)	169 ± 11.6	147 ± 12	-22 ± 17	0.20	176 ± 11.2	136 ± 16	-40 ± 19	0.05	0.87
GSH (μmol/L)	26.3 ± 2.3	16.4 ± 1.9	-9.9 ± 3	0.002	31.8 ± 3.2	19.8 ± 2.5	-12 ± 4.1	0.005	0.69
MPO (ng/mL)	221 ± 6.6	123 ± 12.9	-98 ± 14.2	< 0.001	221 ± 6.7	115 ± 22	-106 ± 23.2	< 0.001	0.61
AOPP (μmol/L)	36.1 ± 5.4	48.4 ± 13.7	12.3 ± 11.2	0.39	26.2 ± 5.5	35.9 ± 7.6	9.7 ± 9.3	0.30	0.57

[1]P values are for the comparison between groups (change from baseline). Plus-minus values are mean ± SE of mean; Continuous parameters were analyzed using the Wilcoxon signed-ranks test and the Wilcoxon rank-sum tests were used for treatment group comparisons.

Table 4 Patients' baseline cytokine values and outcome at 6 mo

Variable	Viusid (n = 30)				Placebo (n = 30)				P value[1]
	Before treatment	After treatment	Change	P value	Before treatment	After treatment	Change	P value	
IL-1α (pg/mL)	3.26 ± 0.7	2.61 ± 0.3	-0.60 ± 0.8	0.04	3.09 ± 0.5	6.62 ± 2.3	3.53 ± 2.4	0.04	0.04
IL-1β (pg/mL)	3.7 ± 0.1	2.9 ± 0.1	-0.8 ± 0.1	< 0.001	4 ± 0.1	3 ± 0.1	-1 ± 0.2	< 0.001	0.24
IL-2 (pg/mL)	17.9 ± 2.3	11.3 ± 0.2	-6.5 ± 2.2	< 0.01	19.9 ± 4.7	12.1 ± 0.8	-7.8 ± 3.9	0.04	0.39
IL-6 (pg/mL)	2.5 ± 0.2	2.1 ± 0.2	-0.4 ± 0.3	0.14	2.1 ± 0.3	2 ± 0.2	-0.1 ± 0.3	0.71	0.24
IL-10 (pg/mL)	2.6 ± 0.6	8.3 ± 4.1	5.7 ± 4	0.04	2.8 ± 4.1	4.1 ± 0.1	1.3 ± 0.2	0.09	0.01
IL-12 (pg/mL)	7.5 ± 1.4	5.1 ± 0.9	-2.4 ± 1.1	0.04	7.6 ± 1.2	6.2 ± 1.5	-1.4 ± 1.4	0.34	0.29
IFN-γ (pg/mL)	1.92 ± 0.2	2.89 ± 0.4	0.97 ± 0.5	< 0.001	1.80 ± 0.1	1.68 ± 0.1	-0.11 ± 0.2	0.70	< 0.0001
TNF-α (pg/mL)	6.6 ± 0.2	7.1 ± 0.4	0.45 ± 0.4	0.26	6.9 ± 0.5	16.2 ± 3.4	9.3 ± 3.1	< 0.01	0.001
TNF-β (pg/mL)	8.5 ± 0.4	5.4 ± 0.1	-3.1 ± 0.5	< 0.001	9.1 ± 0.6	6.3 ± 1.2	-2.8 ± 1.2	0.03	0.21
Cathepsin L (ng/mL)	1.7 ± 0.1	2.9 ± 0.2	1.2 ± 0.2	< 0.001	1.7 ± 0.1	3.1 ± 0.2	1.4 ± 0.2	< 0.001	0.16
GM-CSF (pg/mL)	4.4 ± 0.1	4.6 ± 0.1	0.2 ± 0.2	0.24	4.3 ± 0.1	4.6 ± 0.1	0.3 ± 0.2	0.11	0.60

[1]P values are for the comparison between groups (change from baseline). Plus-minus values are mean ± SE of mean; Continuous parameters were analyzed using the Wilcoxon signed-ranks test and the Wilcoxon rank-sum tests were used for treatment group comparisons.

duced mean levels from 1.80 to 1.68 pg/mL (P = 0.70) in the placebo group (P < 0.0001) (Figure 3B).

Likewise, IL-1α levels slightly decreased from 3.26 to 2.61 pg/mL (P = 0.04) in the patients who received Viusid, as compared with a significant increase from 3.09 to 6.62 pg/mL (P = 0.04) in subjects assigned to placebo (Figure 3C). In addition, IL-10 levels were markedly increased in the patients treated with Viusid (from 2.6 to 8.3 pg/mL, P = 0.04) in contrast to the patients assigned to placebo (from 2.8 to 4.1 pg/mL, P = 0.09) (P = 0.01) (Figure 3D).

Unexpectedly, significant reductions in the mean serum level of IL-1β, IL-2, TNF-β, and cathepsin L were recorded in all patients at 24 wk of treatment in comparison with baseline; however, no significant difference was observed between the groups.

Serum IL-6, IL-12, and GM-CSF concentrations remained unchanged in the patients who received Viusid or placebo as compared with baseline values.

Biochemical results

At 24 wk of treatment, γ-glutamyltransferase (GGT) levels were considerably reduced in comparison with baseline values (Table 5). GGT levels were reduced by 44% (from 72.7 to 40.8 IU/L, P = 0.001) in patients receiving Viusid, as compared with 12% (from 84.1 to 74.4 IU/L, P = 0.56) in the patients assigned to placebo (P = 0.002). Uric acid levels slightly decreased by 23% (from 257 to 198 mmol/L, P = 0.01) in patients receiving Viusid, as compared with 3% (from 276 to 267 mmol/L, P = 0.66) in subjects assigned to placebo.

In both groups, aminotransferase levels were unaffected in comparison with pretreatment values (Table 5).

Safety analysis

Headache and diarrhea were reported in one subject who received Viusid. No laboratory adverse event was reported with the use of Viusid. There was no incidence of discontinuation or dose modification of Viusid secondary to adverse events.

DISCUSSION

Encouraging effects of Viusid on liver histology have been reported in patients with chronic hepatitis C and nonalcoholic fatty liver disease[27,31]; however, pathophysiologic mechanisms for explaining these effects remain unknown. There is a potential hepatoprotective

Table 5 Patients' baseline characteristics and outcome at 6 mo

Variable	Viusid (n = 30)			Placebo (n = 30)			P value[1]
	Before treatment	After treatment	P value	Before treatment	After treatment	P value	
Body mass index (kg/m^2)	25.6 ± 0.7	25.7 ± 0.7	0.92	26 ± 0.7	27.2 ± 0.6	0.08	0.12
Hemoglobin (g/L)	125.8 ± 1.4	125.2 ± 2.1	0.74	132.2 ± 2.7	135.6 ± 3.1	0.09	0.13
Alkaline phosphatase (mmol/L)	217.2 ± 12	206.1 ± 15	0.49	217.1 ± 13	211.4 ± 13	0.69	0.80
ALT (IU/L)	67.6 ± 7.6	65.1 ± 9.9	0.68	67.7 ± 8.4	63.5 ± 6	0.38	0.82
AST (IU/L)	56 ± 7	57.7 ± 9.1	0.81	62.7 ± 7.6	53.9 ± 6.5	0.11	0.24
Fasting plasma glucose (mmol/L)	5 ± 0.1	4.8 ± 0.2	0.17	5.6 ± 0.4	5.4 ± 0.5	0.69	0.91
Creatinine (mmol/L)	79.4 ± 2.8	75.7 ± 2.4	0.06	83.3 ± 3.6	77.7 ± 3	0.17	0.67
γ-glutamyltransferase (IU/L)	72.7 ± 13	40.8 ± 6.1	0.001	84.1 ± 14	74.4 ± 10	0.56	0.002
Albumin (g/L)	47.4 ± 0.5	46.6 ± 0.8	0.43	46.5 ± 0.9	46.1 ± 1	0.72	0.79
Bilirubin (mmol/L)	12.1 ± 0.08	11.9 ± 0.07	0.91	13.8 ± 0.07	14.3 ± 0.08	0.79	0.67
Uric acid (mmol/L)	257 ± 16.8	198 ± 13.8	0.01	276 ± 13.4	267 ± 18	0.66	0.04

[1]P values are for the comparison between groups (change from baseline). Values are expressed as mean ± SE of mean; Continuous parameters were analyzed using the Wilcoxon signed-ranks test and the Wilcoxon rank-sum tests were used for treatment group comparisons.

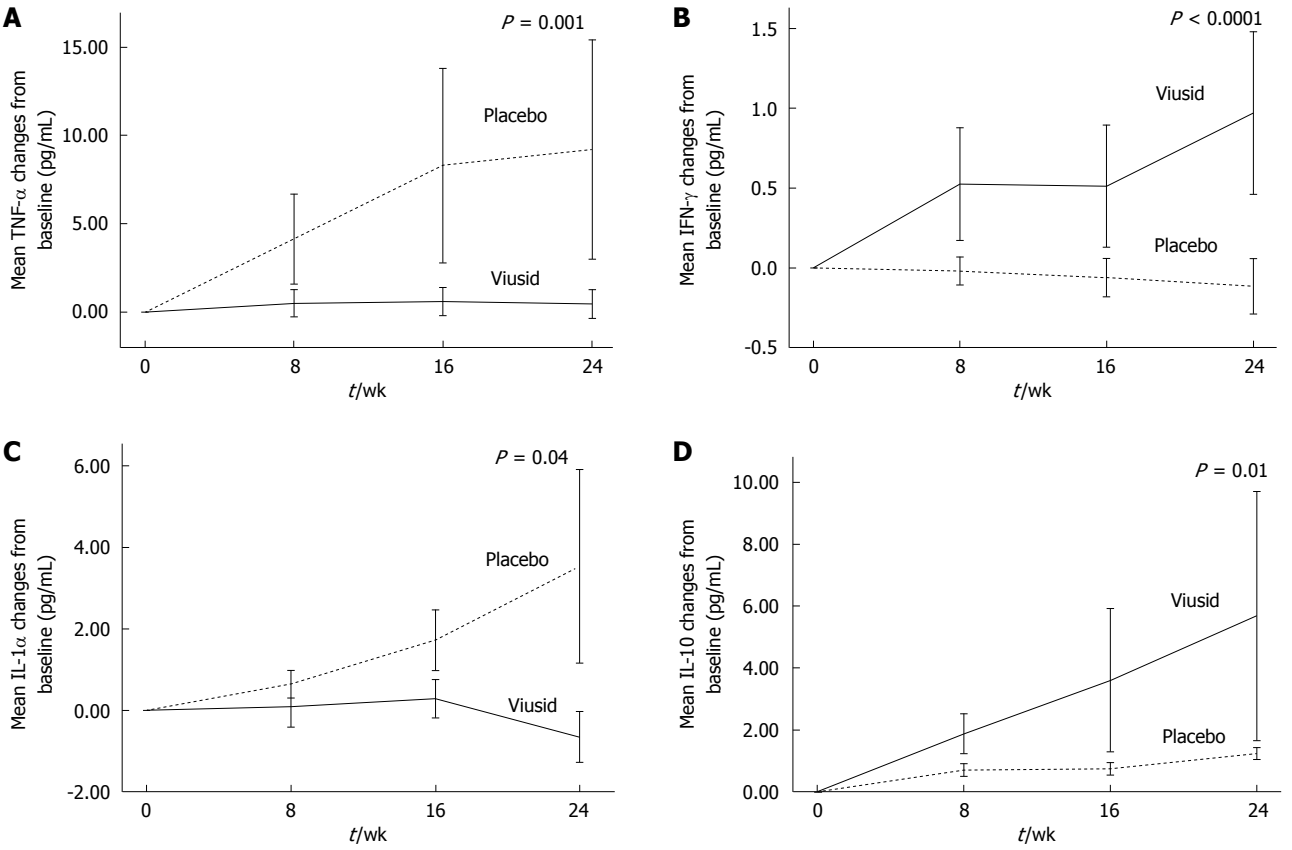


Figure 3 Change in mean serum levels of TNF-α (A), IFN-γ (B), IL-1α (C) and serum IL-10 (D) during the treatment period. Error bars indicate SE. P values for the comparison of the Viusid and the control group at 24 wk. A comparative analysis was performed using Wilcoxon rank-sum test. TNF-α: Tumor necrosis factor-α; IFN-γ: Interferon-γ; IL-1α: Interleukin-1α; IL-10: Interleukin-10.

mechanism in the chemical composition of Viusid that could be explained by the anti-inflammatory and antioxidant properties of its different molecules, such as zinc, glycyrrhizin acid, and ascorbic acid[24-26]. Thus, the aim of this study was to investigate the role of Viusid as an antioxidant and an immunomodulator in patients with CHC who have failed previous antiviral treatment.

Several studies have demonstrated increased production of MDA in CHC, which is an indirect marker for oxidative stress (lipid peroxidation), and its levels have been associated with moderate-to-severe inflammation and fibrosis compared with those patients who had milder disease, suggesting higher oxidative stress with advanced stage of the disease[32,33].

We found that MDA and 4-hydroxyalkenal levels were significantly reduced in the serum of the patients treated with Viusid as compared with placebo, indicating an important effect on lipid peroxidation products. Recent evidence shows that increased lipid peroxidation products, such as 4-hydroxynonenal, play a decisive role

in stellate cell activation and fibrosis progression[34]. Increased 4-hydroxynonenal levels upregulate procollagen and tissue inhibitor of metalloproteinase-1 gene expression. Matrix metalloproteinase-1 plays an important role in degrading collagen; therefore, the inhibition of this enzyme could increase fibrosis. Thus, this provides a putative mechanism to explain in part the effect of Viusid on fibrosis reduction in patients with CHC[27]. Therefore, these results support the rationale for further studies in other forms of liver disease such as alcoholic liver disease and nonalcoholic fatty liver disease.

Encouraging antioxidant effects of ascorbic acid and zinc have been reported recently. Ascorbic acid and zinc supplementation have been associated with a significant reduction of LPO products[35,36].

Surprisingly, a marked reduction of serum antioxidant defense system markers was seen in the whole group during treatment, maybe reflecting the "regression towards the mean" phenomenon, a placebo-induced effect, or poor maintenance of the redox homeostasis status.

Imbalanced Th1- and Th2-type response has been postulated to play an important role in influencing both the persistence of HCV infection and the extent of liver damage[10,37,38]. In this context, IFN-γ and IL-12 are Th1-type cytokines, which are critical in the development of T-cell-mediated immunity, paramount for viral clearance. Thus, an increased production of serum IFN-γ has been strongly linked to self-limited HCV infection[11,12]. Our data suggest an increased production of IFN-γ in the patients treated with Viusid when compared with baseline, maybe suggesting a reestablishment of the T-cell-mediated immunity *via* Th1-type response; however, the underlying mechanisms by which Viusid is able to induce IFN-γ are still poorly understood. On the other hand, IL-10 is a Th2-type response cytokine with a suppressive effect on the generation of Th1-type response cytokines (IFN-γ and IL-12), which have recognized anti-inflammatory and slight proviral effects[13-15]. Our data show that serum IL-10 levels were markedly increased with the administration of Viusid in spite of simultaneously increasing serum IFN-γ levels. IL-10 is known to suppress the secretion of pro-inflammatory cytokines, such as TNF-α, IFN-γ, and IL-12[15,39,40]. In contrast, administration of neutralizing monoclonal antibodies to anti-IL-10 enhances *in vitro* IFN-γ production in HCV patients[41], suggesting that IL-10 levels differ widely between individuals, possibly due to polymorphisms in the promoter region of the *IL-10* gene[42].

Recent data support the hypothesis that long-term IL-10 therapy to treat chronically HCV-infected patients leads to significant histological improvement of inflammation and fibrosis[15].

Our data suggest that increased serum IL-10 levels could be a significant finding to explain the effect of Viusid on histological features (inflammation and fibrosis) in patients with CHC and non-alcoholic fatty liver disease (NAFLD)[27,31].

Recent studies have indicated that glycyrrhizin, an aqueous extract of licorice root and the main ingredient of Viusid, can induce a significant production of IL-10 by liver dendritic cells in concanavalin A-induced hepatitis in mice[43].

Our findings also show that a short course of Viusid slightly reduces IL-1α and stabilizes TNF-α concentrations in patients with CHC. These are markers of inflammation and fibrosis that are elevated in HCV-infected subjects compared with healthy controls[16,17,38]; therefore, their reduction or stabilization could be associated with histological improvement, in particular inflammation and fibrosis.

Viusid has chemical ingredients with recognized immune regulatory properties that would explain in part the effect on cytokine secretion. Zinc supplementation has demonstrated that it can significantly increase IFN-γ and IL-10 secretion and reduce IL-1 and TNF-α secretion in patients with chronic inflammatory diseases[44].

GGT has been regarded as a biomarker of hepatobiliary disease and alcohol consumption/abuse[45]. In addition to liver disease, GGT has been associated with high all-cause mortality, cardiovascular disease incidence and death, and cancer incidence and death[46,47]. Accumulating experimental evidence suggests that GGT is a key mediator in the mechanisms of oxidative stress. GGT-mediated oxidative stress has been reported capable of inducing lipid oxidation, oxidation of protein thiols, alterations of the normal protein phosphorylation patterns, and biological effects such as the activation of transcription factors[48].

In the current study, we found that serum GGT levels were significantly reduced in the patients treated with Viusid in comparison with unchanged levels in the patients assigned to placebo. The mechanism that explains the contribution of Viusid to GGT reduction has not been fully elucidated and further studies are needed to evaluate the clinical impact of long-term Viusid administration in patients with other forms of chronic liver disease, cardiovascular disease, metabolic syndrome, and cancer.

On the other hand, the administration of Viusid did not improve aminotransferase levels during the 24 wk of treatment. In contrast to what has been suggested in two recently published papers[33,37], Viusid without supportive therapy such as antiviral therapy or lifestyle modification for CHC and NAFLD, respectively, is unable to induce improvement in aminotransferase levels, regardless of its proven and significant antioxidant and immunomodulatory capability. Further studies must evaluate higher doses and long-term administration of Viusid to determine its effect on aminotransferase levels in patients with chronic liver diseases.

The main strength of this study is the presence of a concurrent placebo control group allowing adequate comparison between control and experimental groups for outcome measures. A possible weakness of our study is that patients were treated for 24 wk only, and it remains unclear whether some parameters related to oxidative stress and immunological status can be improved beyond 24 wk.

In conclusion, our data suggest that Viusid improves oxidative stress through reduction of lipid peroxidation products and that it has an immunomodulatory

effect on cytokine secretion *via* increased production of IFN-γ and IL-10, decreased production of IL-1α, and stabilized TNF-α secretion in patients with CHC who have failed previous antiviral treatment. Our findings also highlight the limitations in our understanding of the complex mechanism of the host immune response and its interaction with pro-oxidant/antioxidant status.

COMMENTS

Background

The pathogenesis of chronic hepatitis C (CHC) is associated with severe oxidative stress and non-selective immunological disturbance that lead to necroinflammation and the progression of fibrosis. Several trials have suggested that antioxidant and immunostimulant therapies may have a beneficial effect. Two previous clinical studies have reported that the Viusid-related effect on histologic features, especially fibrosis, appears to be associated with antioxidant and/or immunomodulatory properties. However, the putative mechanism of action of Viusid is unknown.

Research frontiers

Peginterferon plus ribavirin is the only therapeutic alternative with proven efficacy in patients with CHC. Unfortunately, sustained virological response is achieved in only approximately 50% of patients. Viusid is a nutritional supplement with documented effects on histological parameters (steatosis, inflammation and fibrosis) in patients with CHC and nonalcoholic fatty liver diseases. It contains different molecules (ascorbic acid, zinc, and glycyrrhizic acid) with recognized antioxidant and immunomodulatory properties. However, is necessary to confirm whether Viusid may have a beneficial effect on oxidative stress and cytokine parameters in patients with CHC.

Innovations and breakthroughs

Recent studies have suggested that the Viusid-related effect on histologic features, especially fibrosis, appears to be associated with antioxidant and/or immunomodulatory properties. All of these effects could modulate the histologic pattern of CHC, especially inflammation and fibrosis, in an attempt to halt disease progression. The identification of the molecular mechanisms of action of Viusid as an immunomodulatory and/or antioxidant product in patients with CHC could support a new strategy of treatment in patients who have failed previous antiviral treatment.

Applications

In current clinical practice, there are no available strategies of treatment for patients with CHC who have failed previous antiviral treatment. Therefore, there is an obvious need for the continuous development of new treatment strategies for CHC. Thus, the administration of compounds with antioxidant and immunomodulatory properties such as Viusid could be a plausible strategy to halt the natural course of the disease, particularly in patients with poor response to antiviral therapy. Further studies are needed to evaluate the clinical impact of the administration of Viusid in patients with end-stage liver disease secondary to CHC.

Terminology

Viusid is a nutritional supplement. It contains different molecules (ascorbic acid, zinc, and glycyrrhizic acid) with recognized antioxidant and immunomodulatory properties. The different compounds in the supplement are activated through a molecular activation principle which strongly increases their biological activity without modifying their structure. With this method the molecules are treated under a determined electric field during a time span calculated beforehand and under certain specific conditions for each kind of molecule.

Peer review

In this report, the authors reported the results of a randomized double-blind and placebo-controlled study to evaluate the effect of Viusid on oxidative stress and cytokine parameters in patients with chronic hepatitis C who had been non-responders to previous antiviral therapy with peginterferon plus ribavirin and infected with genotype 1.

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ORIGINAL ARTICLE

Prophylactic uses of integrin CD18-βA peptide in a murine polymicrobial peritonitis model

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Abstract

AIM: To evaluate the prophylactic properties of integrin CD18-βA peptide in a murine model of abdominal polymicrobial peritonitis and sepsis.

METHODS: Bacterial sepsis was induced in Institute of Cancer Research (ICR) mice by cecal ligation and puncture (CLP) surgery. Inflicted mice were then injected with either sterile saline or CD18-βA peptide intraperitoneally at 2 h after surgery, and were sacrificed at 12 and 24 h after surgery. Blood samples were immediately collected, and analyzed for endotoxin activity and tumor necrosis factor (TNF)-α and interleukin (IL)-6. Lungs and liver were studied for CD45+ leukocyte and CD3 mRNA content. Pulmonary expression of intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM) and E-selectin was also determined.

RESULTS: Intraperitoneal injection of CD18-βA peptide significantly suppressed circulating endotoxin activity ($P < 0.01$) at 24 h, as well as serum levels of TNF-α ($P < 0.05$ at 12 and 24 h) and IL-6 ($P < 0.01$ at 12 h, $P < 0.05$ at 24 h) in CLP-inflicted mice. CD18-βA peptide also abrogated leukocyte infiltration into liver and lungs as unveiled by reduced CD45+ leukocyte and CD3 mRNA contents. Furthermore, the peptide significantly reduced pulmonary expression of VCAM ($P < 0.01$ at 12 h, $P < 0.001$ at 24 h), E-selectin ($P < 0.01$ at 12 and 24 h), and ICAM-1 ($P < 0.01$ at 12 h, $P < 0.001$ at 24 h). These actions of CD18-βA peptide collectively protected septic mice against lethality ($P < 0.01$).

CONCLUSION: CD18-βA peptide is a potent endotoxin antagonist that can protect surgical patients against sepsis-associated lethality.

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Key words: Bacterial endotoxin; Integrin CD18; Inflammation; Biotherapeutic peptide

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INTRODUCTION

Endotoxin [or lipopolysaccharide (LPS)] was first identified by Richard Pfeiffer from the late log phase culture of *Vibrio cholerae*, of which the heat inactivated bacterial

lysates could induce septic shock and death in experimental animals[1,2]. In clinical settings, circulating endotoxin acts as a pivotal initiator in the pathogenesis of bacterial sepsis and signals front-line immune host cells to trigger the "cytokine storm", resulting in systemic inflammatory response syndrome (SIRS)[3]. Given that the deleterious SIRS goes unchecked, patients may develop and die from acute respiratory distress, shock, and multiple organ dysfunction. Severely ill patients in surgical wards and intensive care units are at risk from sepsis-associated death, despite the use of broad-spectrum antibiotics, corticosteroids, and other new drugs like drotrecogin alfa[4]. To resolve the stringent clinical situation, many experimental therapeutics like cultured bone marrow stromal cells[5], cholinergic agonists[6] and lysophosphatidylcholine[7] have emerged as potential therapeutics. Studies on their therapeutic efficacies however still remain in pre-clinical stages.

Our previous finding has identified that leukocyte integrin CD18 βA peptide is able to bind bacterial LPS and to alleviate the pro-inflammatory responses in LPS-stimulated Jurkat cells[8]. In the present study, we evaluated the potential prophylactic use of CD18-βA peptide in a murine model of cecal ligation and puncture (CLP) that resembles abdominal polymicrobial peritonitis and sepsis in humans[9].

MATERIALS AND METHODS

CD18-βA peptide

We designed the CD18-βA peptide basing on the residues 266-318 of CD18 of leukocyte β2 integrins. We cloned the cDNA fragment into pET43.1B vector (Novagen, San Diego, CA, USA), expressed recombinant CD18-βA peptide in *E. coli.* BL21 (DE3) (Novagen), and subsequently purified the resulting protein using ion mobilized affinity chromatography following our published procedure[8,10]. Before we injected CD18-βA peptide into mice, we dialyzed the peptide against sterile phosphate buffered saline. We verified the purity using electrophoresis, and measured the concentration of CD18-βA peptide as described[11].

Polymicrobial CLP peritonitis model in mice

We obtained male Institute of Cancer Research (ICR) mice (30 g in weight) from the AAALAC-accredited Laboratory Animal Unit of the University of Hong Kong (Pokfulam, Hong Kong), and divided them randomly into three experimental groups of equal size. We then maintained all mice in a vivarium on a 12-h light/dark cycle at 21℃. Mice had ad libitum access to a sterilized animal chow and water. We performed all experimental procedures on mice in accordance with the ethical guidelines set forth by the University's Committee on Using Live Animals for Teaching and Research.

We performed CLP as previously described[5] in ICR mice. We anaesthetized mice with an intraperitoneal injection of 50 mg/kg sodium pentobarbital (Nembutal, Rhone Merieux, Pinkenba, QLD, Australia), and opened the anterior abdomen by a 15 mm midline incision. Afterwards, we ligated the exposed cecum non-obstructively with surgical silk, punctured it twice with a 19-gauge needle, and then replaced it back to the peritoneal cavity. For sham-operated mice, we opened the abdomen, but the cecum was neither ligated nor punctured. Finally, we completed CLP surgery by closing the abdomen wall with nylon sutures, and gave no analgesics and antibiotics to mice. At 2 h after the completion of CLP surgery, we injected 0.1 mL sterile saline intraperitoneally into 12 mice, and 0.8 mg/kg CD18-βA peptide (this optimal dosage was prior determined in a pilot study ranging from 0.6-1.2 mg/kg) to another 12 mice. An additional injection of sterile saline or CD18-βA peptide, respectively, was given to mice at 14 h after the completion of CLP surgery. We gave no treatment to the sham-operated mice throughout the experiment. We killed six mice from each of the three experimental groups at 12 and 24 h after CLP. Therefore, mice that were sacrificed at 12 and 24 h received one and two injections of CD18-βA peptide, respectively. We collected blood from the interior vena cava, and harvested lung and liver. We obtained serum from the collected blood by centrifugation at 3000 rpm for 10 min at 4℃. For each harvested lung and liver, we minced it into two pieces that were formalin-fixed and snap-frozen separately.

Survival study

We studied the survival of septic mice receiving different treatments after CLP in another group of ICR mice (n = 8 in each experimental group, in total 24 mice). We performed CLP as previously described, and injected mice intraperitoneally with either sterile saline or CD18-βA peptide at 2, 12 and 24 h after closure of abdomen. Sterile saline was used as a control according to our earlier studies and reported procedures[5,7,12]. We assessed survival after CLP continually for 48 h, and regarded mice that still remained alive beyond the observation period as survivors. Survival data were analyzed using the Kaplan-Meier module in GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA).

Limulus amoebocyte lysate assay of endotoxin activity

We determined the endotoxin activity in circulation using the Limulus amoebocyte lysate (LAL) pyrochrome kit (Associates of Cape Cod Inc, East Falmouth, MA, USA) as previously described[13,14]. All buffers for the LAL assay were prepared using endotoxin-free water according to manufacturer's instructions. We measured the resulting signal spectrophotometrically at 540 nm using a microtiter plate reader (Molecular Devices, Sunnyvale, CA, USA), and each sample was done in triplicate. We finally determined the endotoxin activity from the measured signals using a standard curve with a dynamic range from 0 to 0.623 Endotoxin Units (EU)/mL.

Tumor necrosis factor-α and interleukin-6 enzyme-linked immunosorbent assay

We measured the serum level of tumor necrosis factor (TNF)-α and interleukin (IL)-6 of mice using commercially available enzyme-linked immunosorbent assay kits

that were purchased from Dakewe (Shenzhen, China) and eBioscience (San Diego, CA, USA), respectively, in accordance with instructions previously reported[15,16].

Immunohistochemistry

We followed our published procedures to perform all immunohistochemical staining[17,18]. To study the CD45+ leukocyte content in lungs and liver of mice, we stained tissue paraffin sections with 10 μg/mL monoclonal antibody against CD45 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as described[19]. We stained lung tissues with 20 μg/mL monoclonal antibody against VCAM (20 μg/mL; BD Biosciences, San Jose, CA, USA) and 20 μg/mL monoclonal antibody against E-selectin (BD Biosciences) to study pulmonary expression of the two adhesion molecules. We incubated tissue sections with respective antibodies at 4℃ overnight. After washing, we added a horseradish peroxidase-conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA). We visualized the resulting signals with a liquid DAB substrate kit (Invitrogen), and thereafter, we counterstained tissue sections with hematoxylin (Vector Laboratories, Burlingame, CA, USA).

Real-time polymerase chain reaction

We measured the level of CD3 mRNA in lung and liver of mice after CLP using real-time polymerase chain reaction (PCR). This method provides robust and quantitative measurement on tissue infiltration of inflammatory leukocytes[20]. To study the expression of ICAM-1, VCAM, and E-selectin in lungs, we measured their mRNA levels. We performed RNA extraction, first-strand cDNA synthesis, and real-time PCR in ABI PRISM 7700 sequence detector system (Applied Biosystems Inc., Forest Hill, CA, USA) as previously described[21-23].

Statistical analysis

We performed all statistical analysis using the GraphPad Prism (GraphPad Software Inc.); group means of the study parameters were compared by one-way ANOVA, and P values < 0.05 were considered to indicate statistical significance.

RESULTS

Survival of septic mice

Diagnosis of bacterial sepsis in most clinical scenarios is always delayed, therefore in this study we injected mice intraperitoneally with CD18-βA peptide (0.8 mg/kg) (or sterile saline) at 12-h intervals beginning two hours after the CLP surgery. We first looked at the survival rates after CLP surgery in saline- and CD18-βA peptide-treated mice. A statistically significant ($P < 0.01$) improvement in the survival of mice injected CD18-βA peptide intraperitoneally was noted (Figure 1A); about 40% of mice survived over the 48 h post-operative observation period, and remained alive thereafter despite discontinuation of therapy. In contrast, mice that received saline all died within the 36 post-operative hour period.

Endotoxicity and pro-inflammatory cytokines in circulation

We next investigated whether the beneficial effect of CD18-βA peptide on the survival of septic mice was associated with reductions in endotoxin activity in circulation. To this end, we measured both biological activity of endotoxin and serum levels of TNF-α and IL-6 after we sacrificed mice at 12 and 24 h after CLP surgery and collected blood samples at the interior vena cava. Mice inflicted with CLP surgery showed up-regulation of the circulating endotoxin activity and TNF-α and IL-6 levels in serum samples, which could be remarkably modulated by treatment with CD18-βA peptide but not with saline control (Figure 1B). Intraperitoneal injection of CD18-βA peptide achieved a statistically significant reduction in circulating endotoxin activity ($P < 0.01$) at 24 h, as well as serum levels of TNF-α ($P < 0.05$ at 12 and 24 h) and IL-6 ($P < 0.01$ at 12 h, $P < 0.05$ at 24 h).

CD45+ leukocyte and CD3 mRNA contents in lungs and liver

In view of the fact that infiltrating leukocytes usually injure tissues, we first studied histologically the effect of CD18-βA peptide on CD45+ leukocyte infiltration in lungs and liver of septic mice (Figure 2A). Intense CD45 reactivity was detected in the lungs and liver organs from the placebo group. Strikingly, a bolus of CD18-βA peptide injection drastically diminished CD45+ cells in liver, and almost completely eradicated the CD45+ signals in lungs. Further to confirm this observation, we measured the level of CD3 mRNA that is expressed by infiltrating leukocytes using real-time PCR (Figure 2B). Compared to the sham control, CD3 mRNA levels of lungs and liver were up-regulated by over 10-20 fold by CLP surgery, and these elevations were reduced significantly by treatment with the CD18-βA peptide ($P < 0.05$). Taken together, we suggest that CD18-βA peptide can be used to prevent leukocyte infiltration in bacterial sepsis.

ICAM-1, VCAM and E-selectin expression in lungs

Adhesion molecules facilitate leukocyte trafficking, and are implicated in mediating organ injuries[24]. To study the effect of CD18-βA peptide on adhesion molecule expression, we first determined the pulmonary expression of VCAM and E-selectin by immunohistochemistry (Figure 2C). Intense immunoreactivity toward VCAM and E-selectin was detected in lung paraffin sections prepared from saline-treated septic mice, whereas the corresponding immunoreactivities were markedly diminished from the CD18-βA peptide-treated mice. Expression of ICAM-1, VCAM, and E-selectin mRNA in lungs has also been determined by real-time PCR. Similarly, mRNA levels of the three adhesion molecules surged in lungs of saline-treated septic mice, whereas such elevations were significantly suppressed after CD18-βA peptide treatment: VCAM ($P < 0.01$ at 12 h, $P < 0.001$ at 24 h), E-selectin ($P < 0.01$ at 12 and 24 h), and ICAM-1 ($P < 0.01$ at 12 h, $P < 0.001$ at 24 h) (Figure 2C).

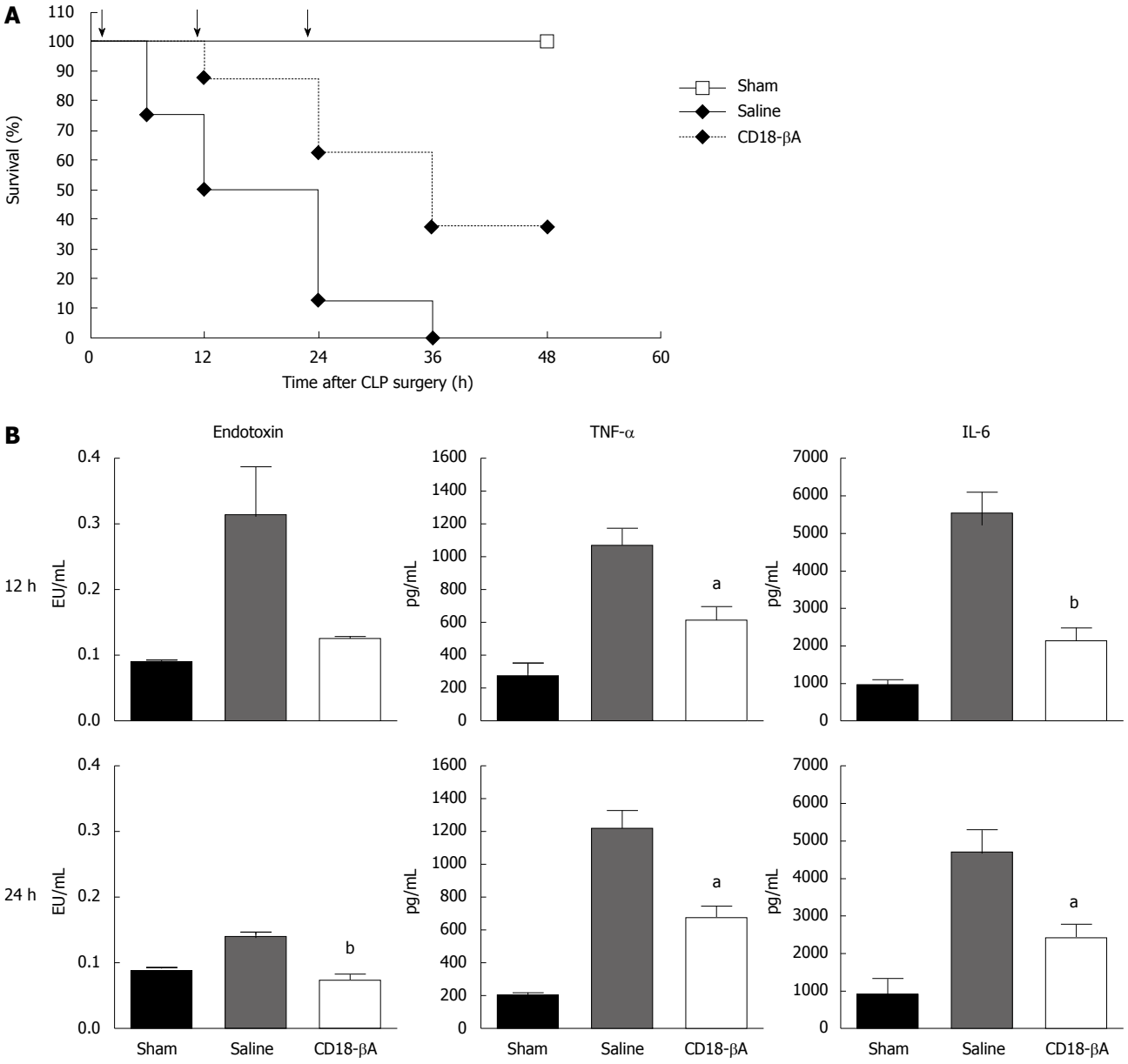
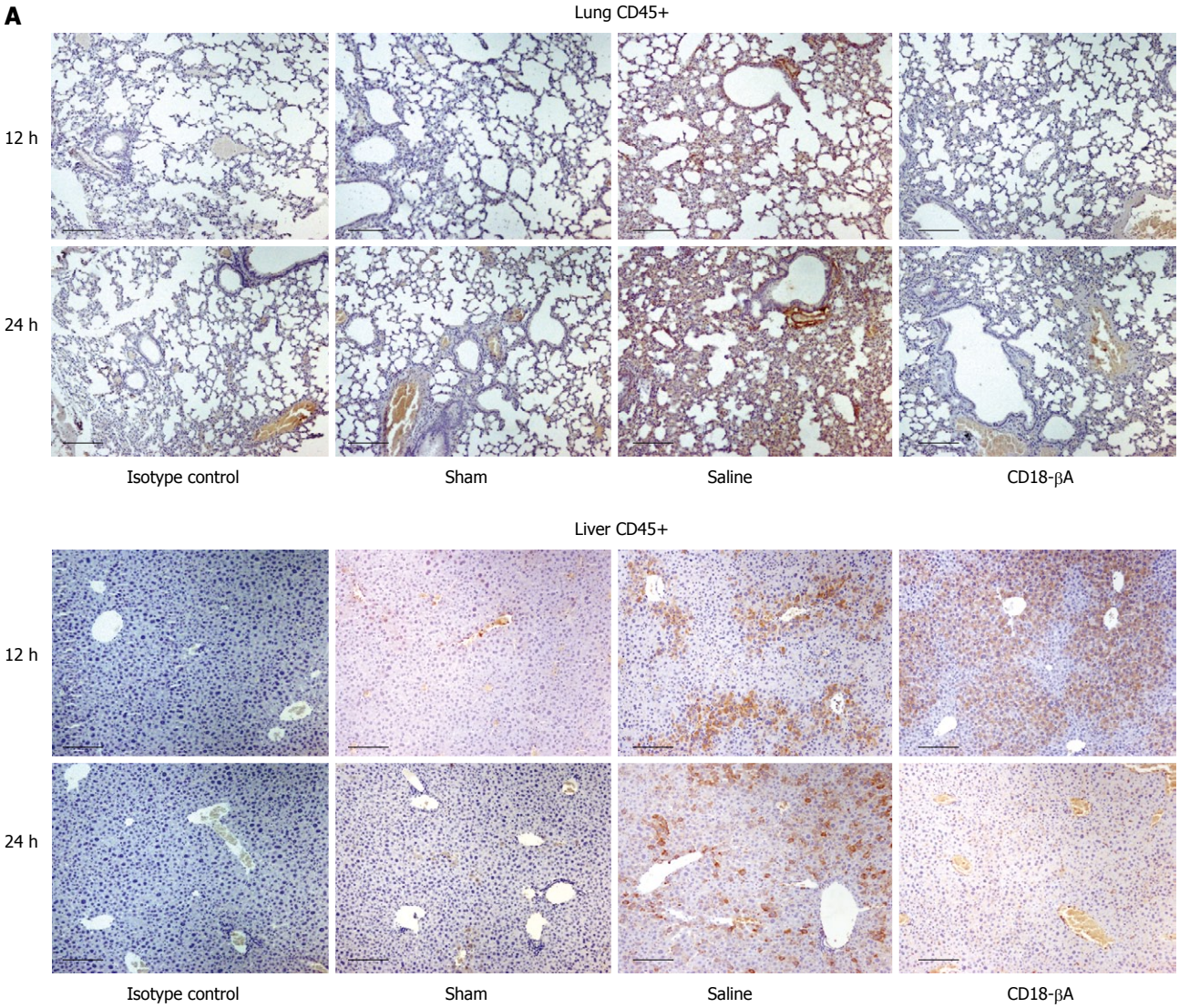


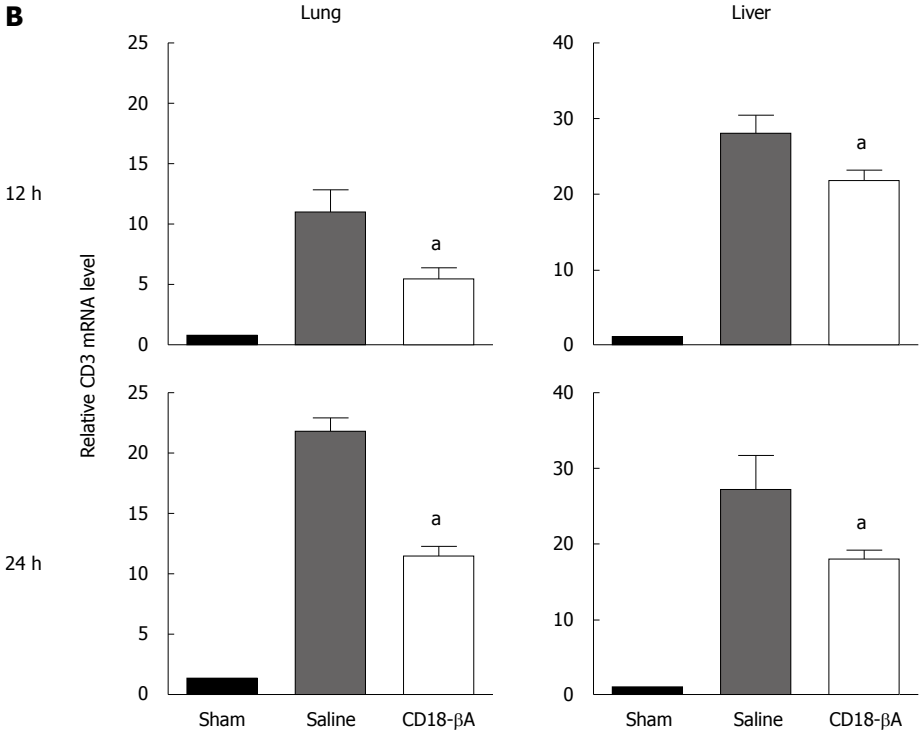
Figure 1 CD18-βA peptide protects mice against cecal ligation and puncture (CLP)-induced lethality and inflammatory responses. A: Mice were injected with either sterile saline or CD18-βA peptide intraperitoneally at 12 h intervals beginning 2 h after CLP (as indicated by arrows), and were monitored continually over 48 h. There was statistically significant improvement in survival of septic shock mice that received CD18-βA injection ($P < 0.01$), compared to those that received saline. n = 8 for each experimental groups; B: Measurement of endotoxin activity and tumor necrosis factor (TNF)-α and interleukin (IL)-6 levels in blood circulation. Injection of CD18-βA peptide achieved significant reductions in endotoxin activity at 24 h, and reductions in TNF-α and IL-6 at 12 and 24 h after CLP. 12 h, n = 5 and 24 h, n = 6 for each experimental group. [a]$P < 0.05$, [b]$P < 0.01$ *vs* saline group (one-way ANOVA). Error bars represent mean ± SD.

DISCUSSION

We illustrated in a stringent murine model of bacterial sepsis that CD18-βA peptide was able to neutralize circulating endotoxin, to reduce serum TNF-α and IL-6 levels, to inhibit leukocyte infiltrating into lungs and liver, and to suppress endotoxin-induced adhesion molecule expression. All these protective actions of CD18-βA peptide might contribute to survival benefits of the animals in the sepsis model.

We found the protection of CD18-βA peptide on septic mice was in some ways impressive. Notably, CD18-βA peptide still afforded protection on septic mice even when it was administered after onset of bacterial sepsis. This is in contrast to many other therapeutic interventions that are incapable of halting the already-initiated super-inflammatory responses in surgical patients. In view of this, CD18-βA peptide can serve as an adjunctive therapeutic particularly for patients who are being challenged by high concentration of endotoxin, which usually results with a delayed diagnosis of bacterial sepsis. One of the possible underlying mechanisms for the effective endotoxin neutralization could be attributed to the presence of an amphipathic helix motif (VGQLAHKLAENNIQPIFA) at the C-terminal end (Figure 3). Such structural moiety favors endotoxin binding and neutralization of


A
Lung CD45+
12 h
24 h
Isotype control
Sham
Saline
CD18-βA
Liver CD45+
12 h
24 h
Isotype control
Sham
Saline
CD18-βA


B
Lung
Liver
12 h
24 h
Relative CD3 mRNA level
25
20
15
10
5
0
40
30
20
10
0
a
Sham
Saline
CD18-βA

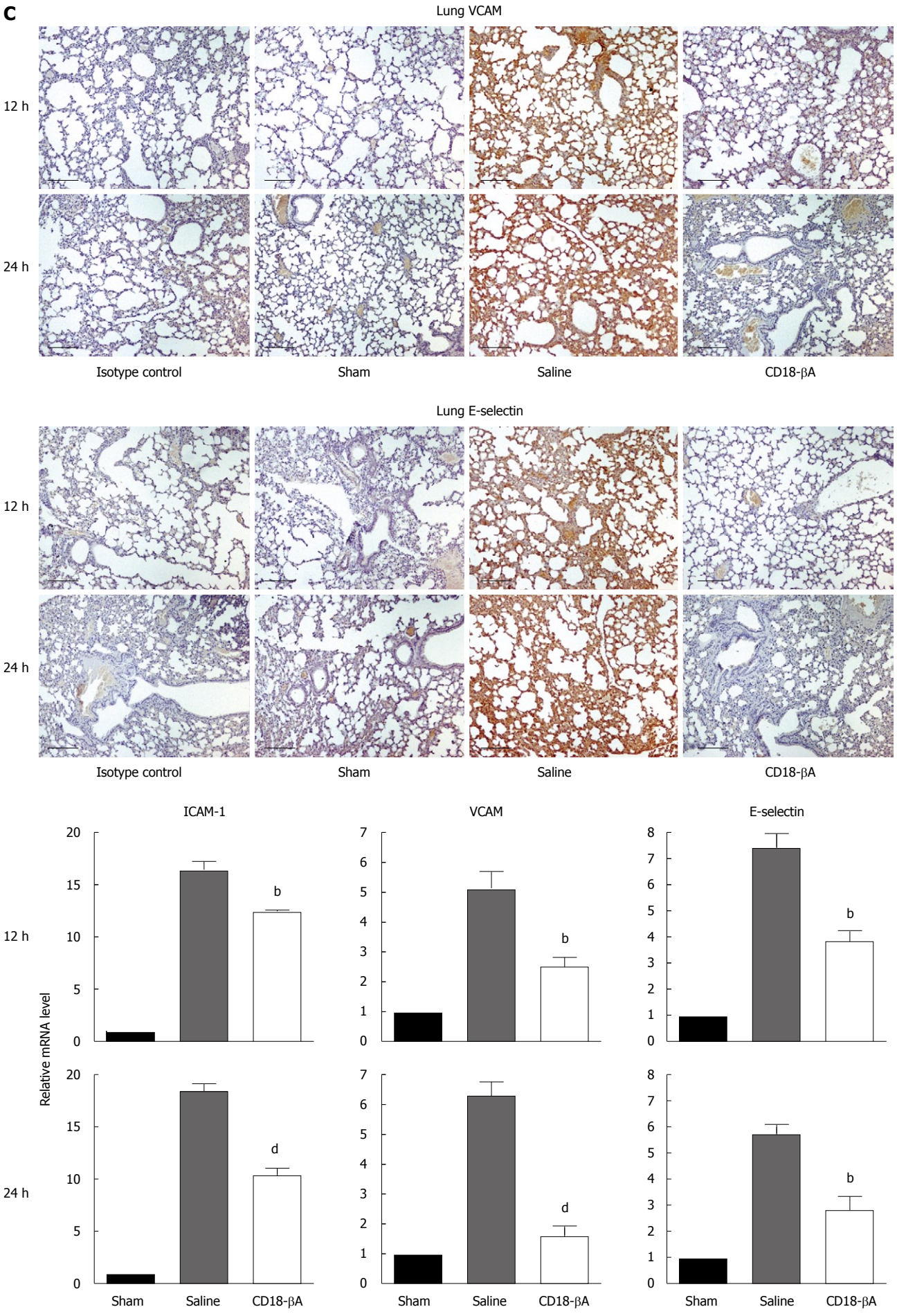

C
Lung VCAM
12 h
24 h
Isotype control
Sham
Saline
CD18-βA
Lung E-selectin
12 h
24 h
Isotype control
Sham
Saline
CD18-βA
ICAM-1
VCAM
E-selectin
Relative mRNA level
12 h
24 h
b
b
b
d
d
b
Sham
Saline
CD18-βA

Figure 2 CD18-βA peptide inhibits CD45+ leukocyte infiltration into lung and liver in CLP-induced mice. A: CD45+ leukocyte content in lung and liver after CLP was visualized by immunohistochemistry. Images are representative of $n = 6$ for each experimental group. Scale bar, 80 μmol/L; B: Real-time polymerase chain reaction (PCR) analysis of CD3 mRNA level in lungs and liver, normalized to β-actin, at 12 and 24 h after CLP. Significant reductions in CD3 mRNA contents were observed in lungs and liver of septic mice that have been treated with CD18-βA peptide. $^{a}P < 0.05$ *vs* saline group (one-way ANOVA); C: Pulmonary expression of adhesion molecules was studied using immunohistochemistry and real-time PCR. Lung sections were stained with VCAM and E-selectin antibody, and images are representative of $n = 6$ for each experimental group. Scale bars, 80 μm. Real-time PCR was employed to study mRNA expression of ICAM-1, VCAM, and E-selectin in lungs. Significant reductions in adhesion molecule mRNA contents were observed in lungs of septic mice that were treated with CD18-βA peptide. $^{b}P < 0.01$, $^{d}P < 0.001$ *vs* saline group (one-way ANOVA). For all real-time PCR study, $n = 6$ for each experimental group at 12 and 24 h. Error bars represent mean ± SD.

endotoxicity[25]. In addition, we have previously shown that the positively charged residues (e.g. arginine, lysine) along the CD18-βA peptide are contributing to the effective binding to LPS of various bacterial strains[8].

We showed CD18-βA peptide is an effective agent to suppress the "cytokine storm" in bacterial sepsis, given that the peptide significantly reduced TNF-α and IL-6 levels in the circulation of septic mice. This aspect of CD18-βA peptide is essential to sepsis treatment because pro-inflammatory cytokines can stimulate phagocytes to release reactive oxygen species, increasing vascular permeability and leading to organ injury[26]. In fact, therapeutic agents like anti-cytokine antibodies and receptor antagonists that are aiming to abolish cytokine functions have long been developed. Unfortunately, none of these agents proved efficacious in protecting sepsis patients[27,28], apparently because of the redundancy of cytokine functions. Unlike these therapeutic agents, use of CD18-βA peptide can circumvent this problem because in our recent study, we have shown CD18-βA peptide could down-modulate the NF-κB-mediated signaling that is central to the release of many pro-inflammatory cytokines.

The clinical utility of CD18-βA peptide as a sepsis therapeutic is further enhanced by its ability to inhibit leukocyte infiltration into lungs and liver, given that leukocyte-mediated injury on these two organs cause the high mortality rate of sepsis patients[29,30]. The inhibition is likely attributed to the suppression of adhesion molecules in lungs. In addition to this mechanism, it was apparent that CD18-βA peptide could block the integrin binding sites on adhesion molecules (e.g. ICAM-1) because CD18-βA peptide is homologous to the CD18 antigen of leukocyte β2 integrins. Indeed, this hypothesis is supported by our early study showing that a recombinant CD18 subunit could inhibit the binding of leukocytes to immobilized adhesion molecules (Luk JM, unpublished observation). We also hypothesized that CD18-βA peptide could antagonize direct activation of leukocytes by bacterial endotoxin as our recent study has illustrated that CD18-βA peptide could prevent the sensing of Jurkat cells by bacterial LPS *in vitro*[8].

Furthermore, a recent *in vivo* animal study of necrotizing enterocolitis has readily demonstrated the potential therapeutic efficacy of CD18-βA peptide against LPS-induced proinflammatory reactions[31]. In that study, rat intestine was distended and then challenged by bacterial LPS, culminating in profound TNF-α release from immune cells, intense leukocyte influx into the intestine, and severe intestinal tissue damage. CD18-βA peptide was able to neutralize the LPS endotoxicity and modulate the above-mentioned deleterious immune responses.

CD18-βA domain

PIDLYYLMDLSYSMLDDLRNVKKLGGDLLRALNEITESGRIGFGSFVDK
TVLPFVNTHPDKLRNPCPNKEKECQPPFAFRHVLKLTNNSNQFQTEVGK
QLISGNLDAPEGGLDAMMQVAACPEEIGWRNVTRLLVFATDDGFHFAGD
GKLGAILTPNDGRCHLEDNLYKRSNEFDYPSVGQLAHKLAENNIQPIFA
VTSRMVKTYEKLTEIIPKSAVGELSEDSSNVVQLIKNAYNKLS

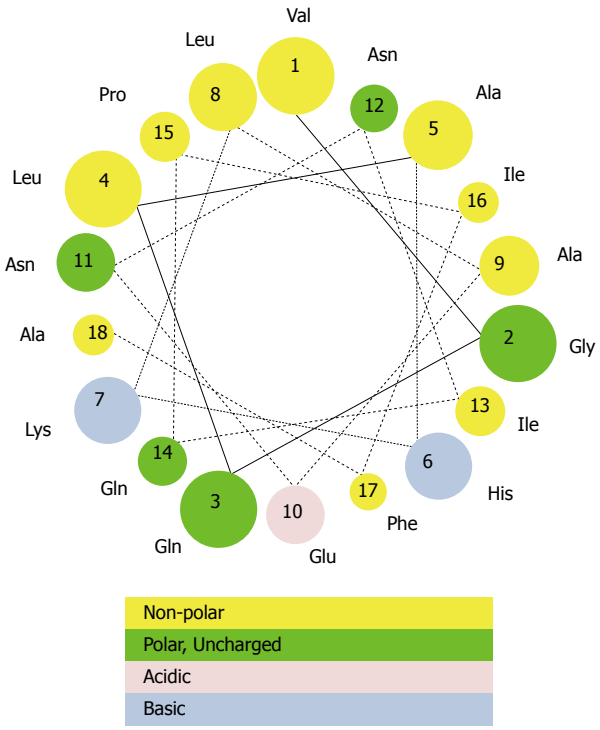


Figure 3 A predicted model of the amphipathic helix sequence at CD18-βA that binds bacterial endotoxin. Shown is the polypeptide sequence of the CD18-βA domain of leukocyte β2 integrin, and the two LPS-binding sites are colored green and red. CD18-βA peptide is derived from the red binding site. The underlined portion of CD18-βA peptide demonstrates a feature of the amphipathic helix, as revealed by the helical wheel diagram.

In summary, CD18-βA peptide is a promising therapeutic for intervention of bacterial sepsis. Unlike anti-LPS antibodies that failed to protect septic patients because of their inability to inhibit pro-inflammatory cytokine releases from immune cells[32], CD18-βA peptide can halt the deleterious "cytokine storm" in bacterial sepsis. To further assess the therapeutic efficacy of CD18-βA peptide, we are going to study the protective action in a porcine model of endotoxemia[33].

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COMMENTS

Background

Patients severely infected with Gram-negative bacteria can die of endotoxemia and sepsis. Indeed, despite the emergence of broad-spectrum antibiotics, corticosteroids, and advanced surgical resuscitative supports, sepsis remains one of the leading causes of death in intensive care units. In this context, many experimental therapeutic agents have been developed over the last few decades. Disappointingly, only a few of these agents were shown to improve survival, and nearly none of them could halt the unchecked inflammatory response of sepsis.

Research frontiers

CD18-βA peptide is a novel endotoxin antagonistic peptide derived from a lipopolysaccharide (LPS)-binding site of leukocyte integrins, and was found able to suppress the stimulation of LPS on leukocytes *in vitro*. The present work is the first published data concerning the use of CD18-βA peptide in the treatment of mice with polymicrobial peritonitis.

Innovations and breakthroughs

The key pathogenesis of sepsis is the super-inflammatory response (or "cytokines storm") that can ultimately lead to multiple organ failure and inevitably death. To date, clinical uses of corticosteroids and antibiotics, and experimental uses of cytokine antibodies all fail to halt the deleterious immunological cascades. In this study, CD18-βA peptide was shown to antagonize endotoxicity in circulation, preventing the pro-inflammatory "cytokines storm" from initiation. This mitigation of the inflammatory response reduced the influx of leukocytes from the bloodstream into lungs and liver, protecting both organs from leukocyte-mediated damage. All these beneficial effects of CD18-βA peptide collectively protected septic mice against death.

Applications

The indication of CD18-βA peptide as a potent endotoxin antagonist promises to be an effective therapeutic agent to alleviate the deleterious superinflammatory responses in patients with acute septic conditions.

Terminology

Polymicrobial peritonitis model is a clinically relevant model that resembles sepsis resulting from endotoxemia. Endotoxin (e.g. LPS) is a structural component of the Gram-negative bacteria cell wall, and once been liberated to circulation, can stimulate the innate immunity to release pro-inflammatory cytokines, culminating in systemic inflammatory response syndrome and sepsis.

Peer review

This is a paper that evaluates the prophylactic properties of integrin CD18, and the results are very important. The authors show integrin CD18 suppressed circulating endotoxin activity and levels of tumor necrosis factor-α and interleukin-6.

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ORIGINAL ARTICLE

Effects of blueberry on hepatic fibrosis and transcription factor *Nrf2* in rats

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Abstract

AIM: To investigate the effects of blueberry on hepatic fibrosis and *NF-E2-related factor 2* (*Nrf2*) transcription factor in rats.

METHODS: Forty-five male Sprague-Dawley rats were randomly divided into control group (A); CCl_4-induced hepatic fibrosis group (B); blueberry prevention group (C); Dan-shao-hua-xian capsule (DSHX) prevention group (D); and blueberry + DSHX prevention group (E). Liver fibrosis was induced in rats by subcutaneous injection of CCl_4 and a high-lipid/low-protein diet for 8 wk (except the control group). The level of hyaluronic acid (HA) and alanine aminotransferase (ALT) in serum was examined. The activity of superoxide dismutase (SOD), glutathione-S-transferase (GST) and malondialdehyde (MDA) in liver homogenates was determined. The degree of hepatic fibrosis was evaluated by hematoxylin and eosin and Masson staining. Expression of *Nrf2* and NADPH quinone oxidoreductase 1 (Nqo1) was detected by real-time reversed transcribed-polymerase chain reaction, immunohistochemical techniques, and western blotting.

RESULTS: Compared with group B, liver indices, levels of serum HA and ALT of groups C, D and E were reduced (liver indices: 0.038 ± 0.008, 0.036 ± 0.007, 0.036 ± 0.005 *vs* 0.054 ± 0.009, $P < 0.05$; HA: 502.33 ± 110.57 ng/mL, 524.25 ± 255.42 ng/mL, 499.25 ± 198.10 ng/mL *vs* 828.50 ± 237.83 ng/mL, $P < 0.05$; ALT: 149.44 ± 16.51 U/L, 136.88 ± 10.07 U/L, 127.38 ± 11.03 U/L *vs* 203.25 ± 31.62 U/L, $P < 0.05$), and SOD level was significantly higher, but MDA level was lower, in liver homogenates (SOD: 1.36 ± 0.09 U/mg, 1.42 ± 0.13 U/mg, 1.50 ± 0.15 U/mg *vs* 1.08 ± 0.19 U/mg, $P < 0.05$; MDA: 0.294 ± 0.026 nmol/mg, 0.285 ± 0.025 nmol/mg, 0.284 ± 0.028 nmol/mg *vs* 0.335 ± 0.056 nmol/mg, $P < 0.05$). Meanwhile, the stage of hepatic fibrosis was significantly weakened ($P < 0.05$). Compared with group A, the activity of GST liver homogenates and expression levels of *Nrf2* and Nqo1 in group B were elevated ($P < 0.05$). The expression level of *Nrf2* and Nqo1 in groups C, D, and E were increased as compared with group B, but the difference was not significant.

CONCLUSION: Blueberry has preventive and protective effects on CCl_4-induced hepatic fibrosis by reducing hepatocyte injury and lipid peroxidation. However, these effects may not be related to the activation of *Nrf2* during long-term of CCl_4.

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Key words: Blueberry; Hepatic fibrosis; NF-E2-related factor 2; NADPH quinone oxidoreductase 1; Glutathione-S-transferase

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INTRODUCTION

Hepatic fibrosis is a common result of chronic injury to the liver[1]. Oxidative stress has recently been recognized as a fundamental factor in the pathological changes observed in various liver diseases[2,3]. Oxidative stress can cause excessive damage to hepatocytes through lipid peroxidation and protein alkylation[4,5]. *NF-E2-related factor 2* (*Nrf2*) is an important transcription factor that regulates antioxidative stress reactions. NADPH quinone oxidoreductase 1 (Nqo1) and glutathione-S-transferase (GST) are the major phase-Ⅱ detoxification enzymes controlled by *Nrf2*[6,7].

Blueberries are flowering plants that belong to *Vaccinium* spp. of the family *Ericaceae*. The Human Nutrition Research Center (Mayer, USA) has performed a series of intensive studies of blueberries. These studies have indicated that blueberries contain anthocyanins, polyphenols and flavonoids, and appear to have the highest antioxidant capacity among the common fruits and vegetables[8,9]. Blueberries may also affect chronic disease through anti-inflammatory and antitumor mechanisms[10,11].

The focus of the present study was to examine the effect of blueberry on hepatic fibrosis and detoxification enzyme systems in rats.

MATERIALS AND METHODS

Ethical approval of the study protocol

All animal studies complied with the Animal Care and Use Guidelines of Guiyang Medical College (Guiyang, China).

Reagents and animal treatments

Forty-five male Sprague-Dawley rats (200 ± 20 g) were obtained from the Experimental Animal Center of Guiyang Medical College [Approval number SCXK (Guizhou) 2002-0001]. Rats were randomly divided into five groups of nine: control group (A); CCl_4-induced hepatic fibrosis group (B); blueberry prevention group (C); Danshao-hua-xian capsule (DSHX) prevention group (D); and blueberry + DSHX prevention group (E).

Except in the control group, liver fibrosis was induced in each group by a complex method[12]. Rats in groups B-E received subcutaneous injections of 40% CCl_4 solution (mixture of pure CCl_4 and peanut oil) and 0.3 mL/100 g twice a week for 8 wk (the first dose was 40% CCl_4 0.4 mL/100 g). Rats were fed a high-lipid/low-protein diet (79.5% corn farina, 20% fat, and 0.5% cholesterol) each day. Rats in group A were fed only a normal diet. At the same time, blueberry juice (1.5 g/100 g per day, *po*, "Rabbiteye Blueberry"; Blueberry production field of Ma-Jiang, Guizhou, China) was processed. Samples were stored at -20℃ until use, and homogenized to prepare blueberry juice (1 mL blueberry juice contained about 2 g of dried blueberries). DSHX (1.0 g/kg per day, *po*; lot number 20081011; DSHX capsules composed of five Chinese herbal medicines, Tetrandrine, *Radix Salviae Miltiorrhizae*, *Radix Paeoniae Rubra*, *Astragalus Membranaceus* and *Ginkgo leaf*; Guiyang Pharmaceutical Company, Guizhou, China) and blueberry juice + DSHX (1.5 g/100 g + 1.0 g/kg, *po*, daily) were given to rats in groups C-E. After 8 wk, there were eight survivors in groups A, B, D and E, and nine in group C.

Rats were killed to collect blood and livers (rats were weighed before killing). The wet liver was weighed. The same part of each liver was removed and fixed in 10% neutral formalin; the remaining portion of liver was stored at -80℃ until use. Rat serum was centrifuged (1500 r/min for 15 min at room temperature) and stored at -80℃ until use.

Histopathology

After fixation in 10% formalin for 24 h, samples were embedded in paraffin. Samples were then cut into 5-μm pieces and mounted on slides. They were stained with hematoxylin and eosin (HE) for histopathological examination, and with fibrosis-specific Masson stain for evaluation of the degree of liver fibrosis[13].

Calculation of the liver index

The liver index was calculated according to the formula[12]: (liver weight/rat weight) × 100%.

Measurement of levels of hyaluronic acid and alanine aminotransferase

Concentrations of hyaluronic acid (HA) (lot number 20090501, Shanghai Institute of Naval Medicine, Shanghai, China) and alanine aminotransferase (ALT) were measured by radioimmunoassay (RIA) or by an automatic biochemical analytic instrument (Siemens Advia 1650, Bensheim, Germany).

Measurement of levels of superoxide dismutase, glutathione-S-transferase and malondialdehyde in liver homogenates

Liver homogenates were prepared. Superoxide dismutase (SOD) content was determined by the xanthine oxidase method; malondialdehyde (MDA) content was tested by the thiobarbituric acid method; and GST content examined by the dinitrobenzene combination method, according to the manufacturer's instructions (lot numbers 20090521, 20090522, and 20090522, respectively; Jiancheng Biologic Co., Nanjing, China).

Real-time reverse transcriptase-polymerase chain reaction analyses

Total RNA was extracted from liver tissues with Trizol reagent (lot number 13827390; Invitrogen, Carlsbad, CA, USA) and purified. This was followed by reverse transcription (lot number 00033699; Fermentas, MBI, Burling-

Table 1 Primer sequences for real-time RT-PCR analyses

Genes	GenBank		
	Number	Forward	Reverse
β-actin	V01217	TCCTCCTGAGCGCAAGTACTCT	GCTCAGTAACAGTCCGCCTAGAA
Nrf2	NM_057152	CCATGCCTTCTTCCACGAA	AGGGCCCATGGATTTCAGTT
Nqo1	NM_017000	CCAATCCTCCACCCACTTGT	GTCCCTCAGCCATTGTTTGAG

RT-PCR: Reverse transcriptase-polymerase chain reaction.

Table 2 Liver index, serum HA, ALT, and SOD, MDA, GST of liver homogenate in different groups (mean ± SD)

Group	*n*	Liver index (relative liver weight)	HA (ng/mL)	ALT (U/L)	SOD (μ/mg)	MDA (nmol/mg)	GST (μ/mg)
A	8	0.03 ± 0.000	351.75 ± 125.16	57.25 ± 6.88	1.76 ± 0.34	0.206 ± 0.052	5.95 ± 1.97
B	8	0.054 ± 0.009[a]	828.50 ± 237.83[a]	203.25 ± 31.62[a]	1.08 ± 0.19[a]	0.335 ± 0.056[a]	7.30 ± 1.26[a]
C	9	0.038 ± 0.008[c]	502.33 ± 110.57[c]	149.44 ± 16.51[a,c]	1.36 ± 0.09[c]	0.294 ± 0.026[a,c]	7.37 ± 0.87[a]
D	8	0.036 ± 0.007[c]	524.25 ± 255.42[c]	136.88 ± 10.07[a,c]	1.42 ± 0.13[c]	0.285 ± 0.025[a,c]	7.35 ± 0.88[a]
E	8	0.036 ± 0.005[c]	499.25 ± 198.10[c]	127.38 ± 11.03[a,c]	1.50 ± 0.15[c]	0.284 ± 0.028[a,c]	7.81 ± 1.16[a]

[a]$P < 0.05$ *vs* group A; [c]$P < 0.05$ *vs* group B. HA: Hyaluronic acid; ALT: Alanine aminotransferase; SOD: Superoxide dismutase; MDA: Malondialdehyde; GST: Glutathione-S-transferase.

ton, ON, Canada) with Moloney murine leukemia virus (MMuLV) reverse transcriptase and oligo (dT)$_{18}$ primers. polymerase chain reaction (PCR) primers were supplied by Dr. Jie Liu (American National Cancer Institute at the National Institute of Environmental Health Sciences (NC, USA) (Table 1). The SYBR Green DNA PCR kit (lot number 0804104; Applied Biosystems, Foster City, CA, USA) was used for real-time reverse transcriptase (RT)-PCR analyses. The cycle time (C_t) values of the genes of interest were first normalized with β-actin from the same sample. Relative differences between groups calculated and expressed as relative increases, setting the control as 100%.

Immunohistochemical analysis

After de-paraffinization, rehydration, and antigen unmasking by heat treatment, histological sections were placed in 3% H_2O_2 for 10 min. They were then incubated with anti-*Nrf2* (lot number K2008; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-Nqo1 (lot number B1508; Santa Cruz Biotechnology) antibody (1:100) overnight at 4℃. Samples were then processed using an EnVision kit (lot number 10N1775A; Dako, Denmark) according to the manufacturer's protocols. PBS was used as the negative control. Yellow material in the cytolymph/nucleus was considered to be a positive cell. Five microscopic fields (× 400 magnification) were randomly chosen per slice, and the number of cells per field counted.

Western blotting

Protein in the total and cytoplasm were extracted using the Bradford method quantified using kits (lot numbers 090310 and 090401; Nanjing Kaiji Biological Engineering Research Institute, Beijing, China). Forty micrograms of total extract (*Nrf2*)/cytoplasm extract (Nqo1) were boiled in an equal volume of 2 × SDS electrophoresis sample buffer for 5 min before 12% SDS-PAGE. After electrophoresis, proteins were transferred to polyvinylidene fluoride membranes (lot number K7KN9080G; Millipore, Billerica, MA, USA). Membranes were blocked with 5% fat-free dry milk-TBST buffer for 1 h at room temperature, and then washed in TBST buffer. Membranes were incubated with a 1:1000 dilution of primary antibody of *Nrf2* and Nqo1 overnight. On the second day, membranes were washed, followed by incubation with a 1:5000 dilution of horseradish peroxidase secondary antibody (lot numbers D2409 and G1009; Santa Cruz Biotechnology) for 1 h. Transferred proteins were visualized with an electrochemiluminescence detection kit (lot number 09065A2; Millipore) and short exposure of the membrane to X-ray films. β-actin (lot number 028K4826; Sigma-Aldrich, St. Louis, MO, USA) was used as an internal reference.

Statistical analysis

Data analyses were carried out using SPSS 11.5 software (Chicago, IL, USA). Quantitative data were expressed as mean ± SD and subjected to one-way analysis of variance. Ordinal data were analyzed by Ridit analysis. $P < 0.05$ was considered significant.

RESULTS

Effect of blueberry on the liver index and serum concentrations of HA and ALT

The liver index and serum concentrations of HA and ALT in group B increased greatly compared with those in group A ($P < 0.05$). Compared with group B, the liver index and serum concentration of HA and ALT in groups C-E were clearly reduced ($P < 0.05$) (Table 2).

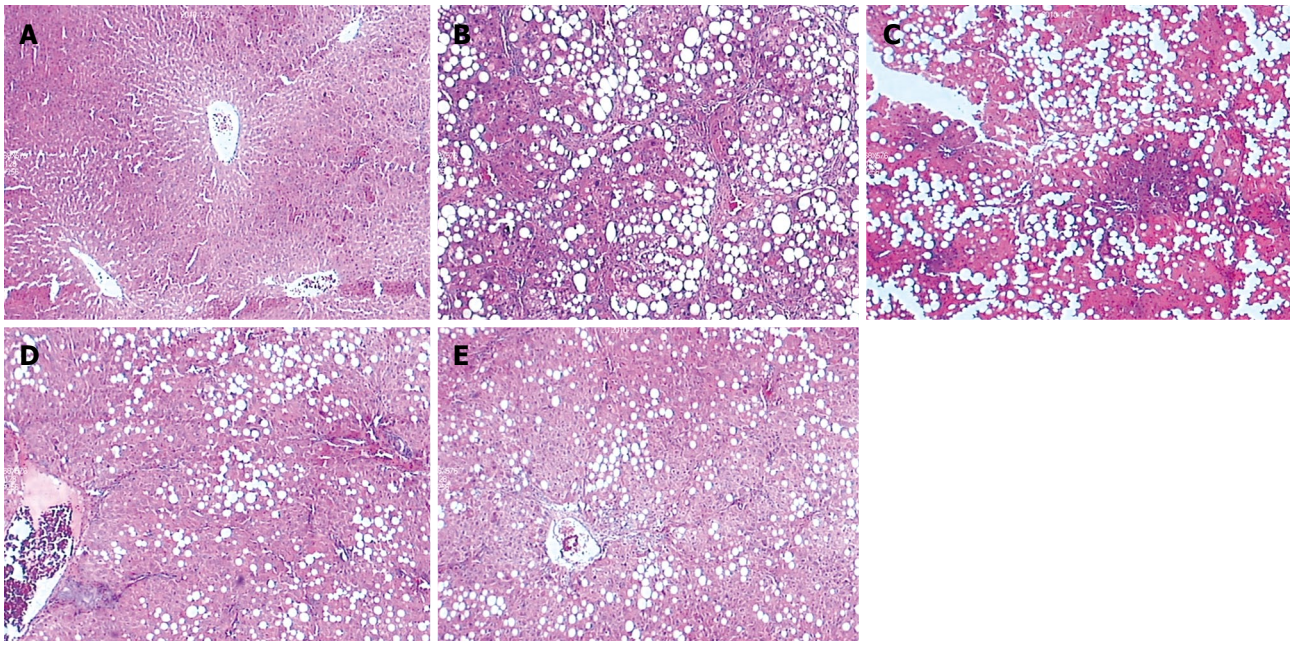


Figure 1 Liver tissue in each group of rats (HE staining, × 40 magnification). A: Light microscopy showing normal liver tissue in the control group; B: Marked fibrosis in the model group; C-E: Pathological change in the treatment group was milder compared with that in the model group.

Table 3 Degree of hepatic fibrosis in each group of rats

Group	*n*	Degree of hepatic fibrosis							Mean rank
		0	1	2	3	4	5	6	
A	8	5	3	0	0	0	0	0	4.5
B	8	0	0	0	0	1	6	1	34.25[a]
C	9	0	0	3	2	2	2	0	23.00[a,c]
D	8	0	0	4	1	1	1	1	22.38[a,c]
E	8	0	0	4	1	2	1	0	20.63[a,c]

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

Effect of blueberry on levels of SOD, MDA and GST in liver homogenates

The level of SOD in liver homogenates was significantly higher and that of MDA significantly lower in groups C-E compared with those in group B ($P < 0.05$) (Table 2). The level of GST was significantly higher in group B than that in group A ($P < 0.05$). The activity of GST in groups C-E was increased as compared with group B, but the differences were not significant.

Effects of blueberry on the degree of hepatic fibrosis

After HE and Masson staining, hepatocytes in group A had a radial array with the central vein, and regenerating collagen fibers were absent. The lobular structure of group-B hepatocytes was destroyed, and the hepatic plates were disordered with diffuse, fatty degeneration. Collagen fibers expanded into the hepatic parenchyma, and fibrous septa surrounding and separating the normal lobules formed. Pseudolobules were observed in a few samples, and numerous inflammatory cells infiltrated in the portal area and fibrous septa. The degree of hepatic fibrosis in group B was significantly more compared with that in group A ($P < 0.05$).

The hepatic fibrosis in groups C-E was alleviated; the fibrous septa were thinner than those in group B ($P < 0.05$). Diffuse fatty degeneration in hepatocytes was found in some samples. Bridging fibrosis, a few inflammatory cells infiltrated into the portal area, fibrous septa, and pseudolobules were also found in some samples. More detailed information about the degree of hepatic fibrosis in each group is shown in Table 3 and Figure 1.

Effect of blueberry on gene and protein expression of Nrf2 and Nqo1

Real-time RT-PCR showed that *Nrf2* and Nqo1 mRNA expression was significantly higher in group B than group A ($P < 0.05$); and expression of *Nrf2* and Nqo1 mRNA in groups C-E was increased as compared with group B, but the differences were not significant. Immunohistochemical and western blotting assays showed similar protein expression of *Nrf2* and Nqo1 (Table 4 and Figures 2-4).

DISCUSSION

The present work demonstrated that blueberry has a therapeutic effect on CCl_4-induced hepatic fibrosis in rats. There was no difference in therapeutic effect among the blueberry, DSHX, and blueberry + DSHX groups. DSHX is a mixed preparation composed of five traditional Chinese herbal medicines. We have previously reported that DSHX is effective in preventing hepatic fibrosis[12,14,15].

Reactive oxygen species and oxidative stress have an important role in the development of hepatic fibrosis. A central role in the defense against oxidative stress has been attributed to the transcription factor *Nrf2*, which

Table 4 Expression of *Nrf2* and Nqo1 in each group (mean ± SD)

Group	*n*	Relative amount of mRNA		Protein expression (%)		Protein level	
		Nrf2	Nqo1	*Nrf2*	Nqo1	*Nrf2*	Nqo1
A	8	46.86 ± 6.49	13.38 ± 4.37	57.50 ± 26.14	15.25 ± 3.62	0.21 ± 0.03	0.06 ± 0.03
B	8	254.88 ± 55.96[a]	68.38 ± 12.61[a]	78.75 ± 15.29[a]	42.50 ± 16.26[a]	0.30 ± 0.06[a]	0.16 ± 0.02[a]
C	9	333.44 ± 90.53[a]	72.78 ± 15.94[a]	85.11 ± 13.65[a]	54.44 ± 15.09[a]	0.34 ± 0.08[a]	0.18 ± 0.03[a]
D	8	277.00 ± 67.38[a]	70.63 ± 20.17[a]	80.00 ± 21.38[a]	50.63 ± 18.98[a]	0.33 ± 0.09[a]	0.17 ± 0.04[a]
E	8	345.00 ± 72.60[a]	77.88 ± 27.50[a]	87.25 ± 14.16[a]	58.13 ± 15.80[a]	0.35 ± 0.09[a]	0.18 ± 0.02[a]

[a]$P < 0.05$ *vs* group A.

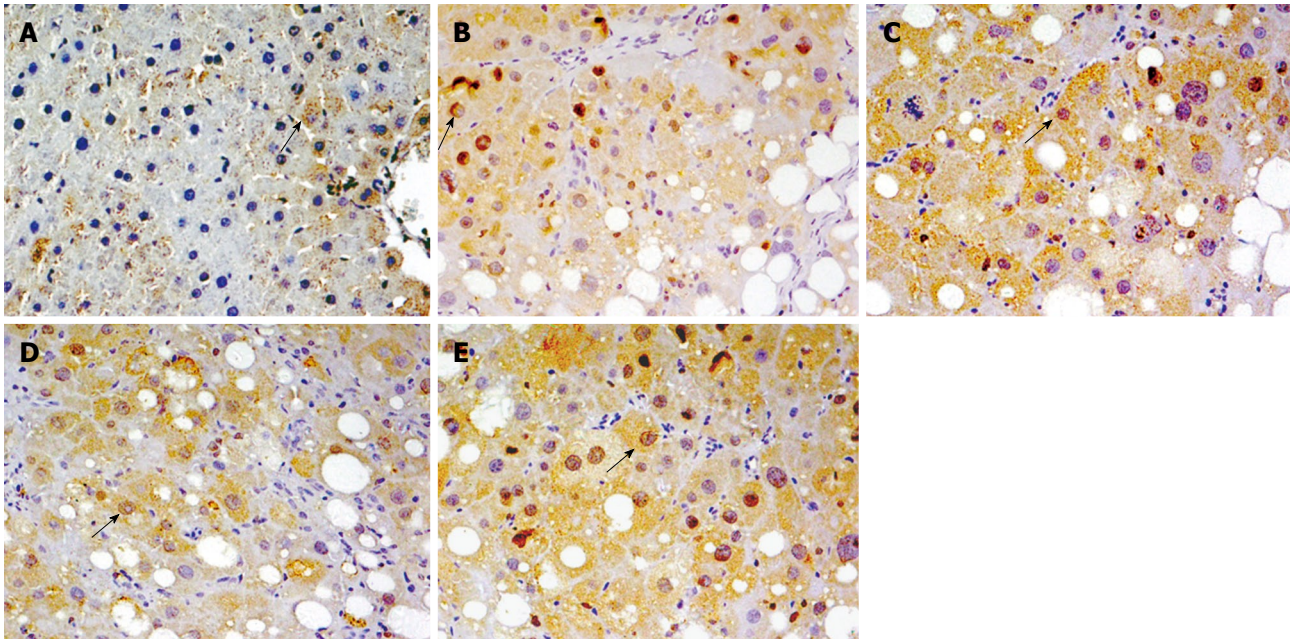


Figure 2 Expression of Nrf2 protein in rat livers (immunohistochemical, × 400 magnification, arrows indicate cytolymph/nucleus-positive cells). A: Cytolymph-positive cells in the control group; B-E: More cytolymph/nucleus-positive cells in the model group and each treatment group compared with the control group.

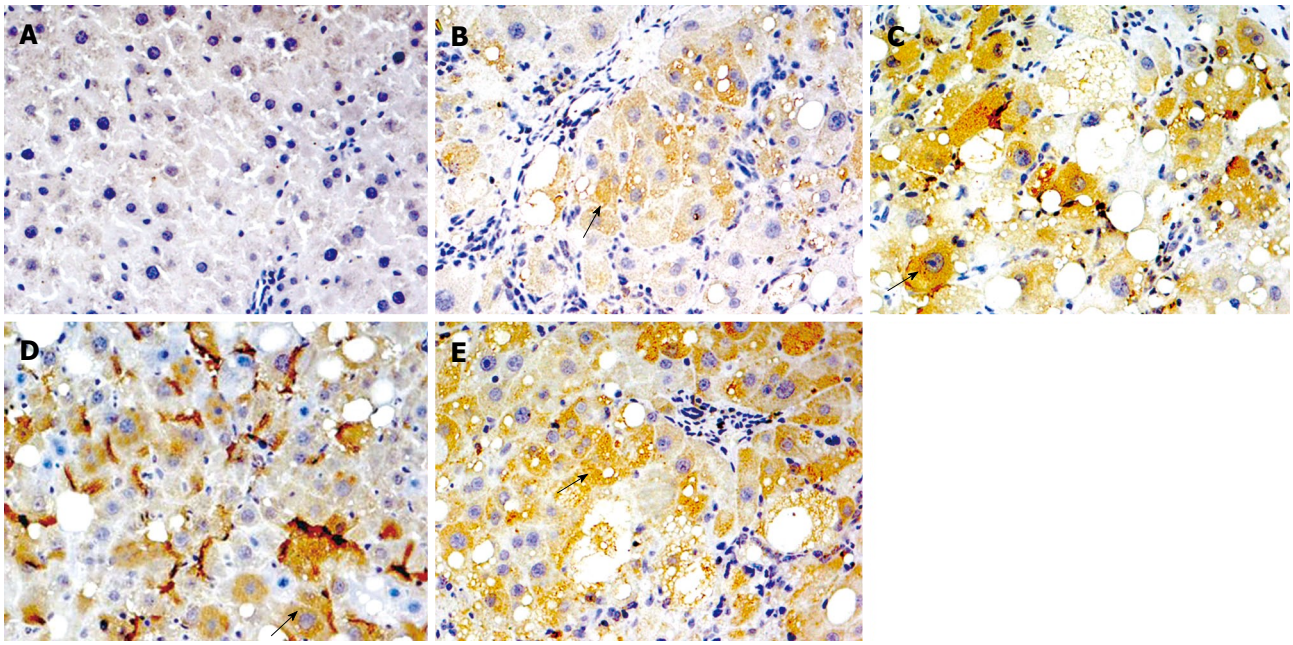


Figure 3 Expression of Nqo1 protein in rat livers (immunohistochemical, × 400 magnification, arrows indicate cytolymph-positive cells). A: Few cytolymph-positive cells in the control group; B-E: More cytolymph-positive cells in the model group and each treatment group compared with the control group.

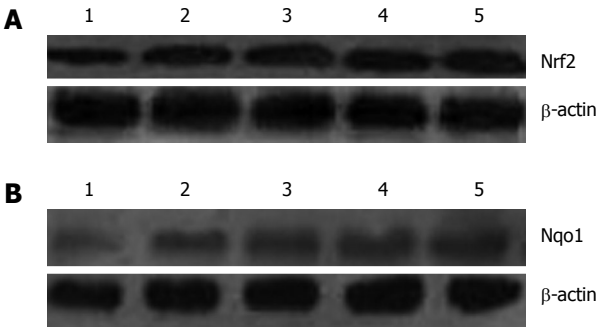


Figure 4 Expression of Nrf2 (A) and Nqo1 (B) protein assessed by Western blotting. Lane 1: Control group; Lane 2: Model group; Lane 3: Blueberry prevention group; Lane 4: DSHX prevention group; Lane 5: Blueberry + DSHX prevention group. The molecular weight of Nrf2 is 57 kDa, the molecular weight of Nqo1 is 31 kDa.

belongs to the basic region-leucine zipper family. In health[16], *Nrf2* localizes in the cytoplasm, where it interacts with the actin-binding protein Kelch-like ECH associating protein 1 (Keap1), and is rapidly degraded by the ubiquitin-proteasome pathway. Signals from reactive oxygen species or electrophilic insults target the *Nrf2*-Keap1 complex, which dissociates *Nrf2* from Keap1. Stabilized *Nrf2* then translocates to the nuclei, and promotes the expression of many antioxidant proteins and phase-Ⅱ detoxifying enzymes such as Nqo1, GST and heme oxygenase-1 (HO-1)[17,18]. Activation of *Nrf2* may be a novel strategy to prevent or ameliorate toxin-induced liver injury and fibrosis[17]. Curcumin has been shown to attenuate dimethylnitrosamine-induced liver injury in rats through *Nrf2*-mediated induction of HO-1[19]. Aleksunes *et al*[20] have proposed that *Nrf2* is crucial for cytoprotection by coordinately activating detoxification genes and preventing the pathogenesis of liver disease.

Blueberry is a small round berry and has high cellular antioxidant activity. Increasing fruit consumption is a logical strategy to increase antioxidant intake and reduce oxidative stress[21]. Studies have suggested that fractions from blueberries may alter phase-Ⅱ detoxification enzymes in cell culture[22]. Boateng *et al*[23] and Reen *et al*[24] have reported significant increases in the activity of phase-Ⅱ enzymes GST-α and NADPH quinine reductase in rats after supplementation with freeze-dried berries. Blueberry and probiotics exert protective effects on acute liver injury induced by d-galactosamine and lipopolysaccharide. They reduce the injury to hepatocytes (including inflammation and secretion of pro-inflammatory cytokines), improve barrier functions, and have antioxidant activity[25]. Proanthocyanidin isolated from blueberry leaves may be used against hepatitis C virus by inhibiting viral replication[26].

Our previous work has showed that blueberries can increase the level of mRNA expression of *Nrf2*, Nqo1, and HO-1 in rat liver if given orally for 21 d, and have good protective effects against acute hepatic injury in rats[27,28]. Blueberries can also increase expression of *Nrf2* and HO-1 in primary hepatic stellate cells (data not shown). The present study tested the hypothesis that blueberries may have preventive protective effects on CCl_4-induced hepatic fibrosis, and that these effects may be related to the activation of phase-Ⅱ enzymes in rat livers.

We found that, in the prevention groups, liver weight and serum levels of HA and ALT were significantly reduced, and liver fibrosis was alleviated. The level of SOD in liver homogenates increased and MDA level decreased. These results indicated that blueberry had certain therapeutic effects on CCl_4-induced hepatic fibrosis in rats through inhibition of liver inflammation and lipid peroxidation. There was no statistically significant difference in therapeutic effect among the blueberry, DSHX and blueberry +DSHX groups, which indicates that blueberry and DSHX did not act in synergy.

Compared with the normal group, GST activity and expression of *Nrf2* and Nqo1 were increased in the model group ($P < 0.05$). GST activity and expression of *Nrf2* and Nqo1 were also increased in the blueberry, DSHX and blueberry + DSHX groups compared with the model group (particularly in the blueberry and blueberry + DSHX group), but these increases were not significant at the concentrations of blueberry used ($P > 0.05$).

In summary, in the present study, blueberries possessed a therapeutic effect on CCl_4-induced hepatic fibrosis in rats through inhibiting liver inflammation and lipid peroxidation, and may be not related to the induction of phase-Ⅱ enzymes through the activation of *Nrf2* in rat liver during long-term of CCl_4. More detailed study is needed.

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COMMENTS

Background

Conventional drugs used in the treatment of liver diseases inevitably have side effects. An increasing number of natural substances have been studied to explore if they have protective effects on the liver. Blueberries have unique effects on human retinal, brain and tumor cells, but reports about the effects of blueberries on liver diseases are lacking.

Research frontiers

Reactive oxygen species and oxidative stress have an important role in the development of hepatic fibrosis. Blueberries have high cellular antioxidant activity. Recent reports have suggested that proanthocyanidin isolated from blueberry leaves can be used against hepatitis C virus by inhibiting viral replication.

Innovations and breakthroughs

The present study showed that blueberries have therapeutic effects on CCl_4-induced hepatic fibrosis in rats, through inhibition of liver inflammation and lipid peroxidation. This protective effect may not be related to the activation of NF-E2-related factor 2 in rat livers.

Applications

Increasing consumption of blueberries is a reasonable strategy to increase antioxidant intake, and may lead to a reduced risk of hepatic disease.

Peer review

The authors present some data from their research on the effectiveness of blueberries on liver fibrosis induced in laboratory animals. Readers of the journal may find it interesting to read the data.

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BRIEF ARTICLE

Diarrhea in neonatal intensive care unit

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Abstract

AIM: To investigate the frequency, etiology, and current management strategies for diarrhea in newborn.

METHODS: Retrospective, nationwide study involving 5801 subjects observed in neonatal intensive care units during 3 years. The main anamnesis and demographic characteristics, etiology and characteristics of diarrhea, nutritional and therapeutic management, clinical outcomes were evaluated.

RESULTS: Thirty-nine cases of diarrhea (36 acute, 3 chronic) were identified. The occurrence rate of diarrhea was 6.72 per 1000 hospitalized newborn. Etiology was defined in 29 of 39 newborn (74.3%): food allergy (20.5%), gastrointestinal infections (17.9%), antibiotic-associated diarrhea (12.8%), congenital defects of ion transport (5.1%), withdrawal syndrome (5.1%), Hirschsprung's disease (2.5%), parenteral diarrhea (2.5%), cystic fibrosis (2.5%), and metabolic disorders (2.5%). Three patients died due to complications related to diarrhea (7.7%). In 19 of 39 patients (48.7%), rehydration was performed exclusively by the enteral route.

CONCLUSION: Diarrhea in neonates is a challenging clinical condition due to the possible heterogeneous etiologies and severe outcomes. Specific guidelines are advocated in order to optimize management of diarrhea in this particular setting.

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Key words: Chronic diarrhea; Congenital diarrhea; Food allergy; Oral rehydration solution; Rotavirus

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INTRODUCTION

Diarrhea and neonatal age are two major factors responsible for pediatric mortality worldwide[1,2]. The neonate has increased susceptibility to complications related to diarrhea due to immaturity of the systems that regulate fluid homeostasis and immunologic response[3]. Early diagnosis and timely treatment are crucial because diarrhea in neonates may rapidly lead to life-threatening dehydration and malnutrition[4,5]. Clinical and epidemiologic studies defining severity and etiology are needed in order

to improve diagnostic and therapeutic approaches for neonatal diarrhea. Starting with these considerations, the Working Group on Intestinal Infections of the Italian Society of Pediatric Gastroenterology, Hepatology and Nutrition (SIGENP), on behalf of the Italian Society of Neonatology (SIN), designed a nationwide study aimed at investigating the frequency, etiology, clinical features, nutritional management, therapeutic approach, and outcomes of diarrhea observed in hospitalized neonates.

MATERIALS AND METHODS

A multicenter, retrospective study was planned. The study design and aims were presented and discussed during two meetings of the SIGENP and the SIN. We invited the participation of the chiefs of neonatal intensive care units (NICUs) of urban children's hospitals, university medical centers or large community hospitals, observing at least 100 newborns per year and having the following diagnostic facilities: determination of fecal electrolyte concentrations, full microbiological examination, food allergy tests, gastrointestinal endoscopy and histology, metabolic tests and genetic counselling. The neonatologists operating in the participating centers were invited to review the data of 3 consecutive years (i.e. 2000-2002). Inclusion criteria were: (1) age at hospitalization $\leqslant$ 28 d; (2) gestational age at birth $\geqslant$ 24 wk; (3) clinical chart and hospital records available for review; and (4) presence of diarrhea, defined on the basis of increased frequency and watery consistency of stools along with dehydration. This definition, which was adopted in a previous study of neonates[4], is in accordance with the traditional definition employed in pediatric gastroenterology[6] and to the more recent guidelines for the management of acute gastroenteritis of the European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN)/European Society for Paediatric Infectious Diseases (ESPID)[7]. Data were recorded in a specific reporting form made up of 5 sections: (1) demographic characteristics: sex, gestational age, birth weight, age at hospitalization and at diarrhea onset; (2) anamnesis: intrauterine growth restriction, polyhydramnios, risk for allergy according to American Academy of Pediatrics (AAP) guidelines[8], familiarity for chronic diseases including diarrhea, surgery, diet before diarrhea onset; (3) characteristics of diarrhea: number of bowel movements, stool consistency, presence of blood and mucus in the stools, severity of dehydration, complications, development of chronic diseases, diarrhea duration and presence of other intestinal or extra-intestinal symptoms; (4) etiology of diarrhea; and (5) nutritional and therapeutic management, clinical outcomes.

To better define the study population and to establish the diarrhea occurrence rate, the number of hospital admissions and the main demographic characteristics of all subjects observed during the study period, in each participating NICU, were also evaluated. Diarrhea was classified as acute or chronic if it lasted < 14 d or > 14 d, respectively, according to the definition of the World Health Organization[9,10]. The study protocol was approved by the Ethics Committee of our institution. No competing interest was declared by the investigators involved in the study.

Statistical analysis

Patients were classified according to the duration of diarrhea into the acute and chronic groups. For continuous variables, the *t*-test for equality of means was used. For categorical variables, the χ^2 test and Fisher's exact test were used. Possible correlations between the duration of diarrhea and gestational age, birth weight, sex, number of bowel movements, antibiotic use, age at diagnosis and severity of dehydration were investigated by linear regression analysis. The level of significance for all statistical tests was 2-sided $P < 0.05$. Statistical analysis was performed using SPSS software (Version 14.0 for Windows, SPSS Inc., Chicago, IL, USA).

RESULTS

Sixteen NICUs were invited to participate in the study and 7 accepted. The clinical charts of 5801 cases were reviewed, and 39 cases of diarrhea were reported. The occurrence rate of diarrhea was estimated at 6.72 per 1000 hospitalized newborn. The number of cases observed during the study period was: 12 in the first, 14 in the second and 13 in the third year of the study. Diarrhea was the main cause of hospital admission in 14 of 39 subjects (35.9%). Diarrhea was classified as acute in 36 patients (92.3%) and chronic in 3 neonates (7.7%). The etiology of diarrhea was identified in 29 of 39 patients (74.3%) (Table 1).

A diagnosis of cow's milk allergy (CMA) was reported in 8 cases. Five of these cases were considered to be at risk for allergic diseases according to the AAP definitions[8]. In patients with CMA, diarrhea was associated with eczema or vomiting in 6 and in 5 subjects, respectively. Specific IgE titers against milk proteins were positive in 6 of 8 patients (> 5.0 kU/L)[11]. In each case of CMA, the diagnosis was confirmed by observation when an antigen elimination diet with an extensively hydrolyzed casein formula (Nutramigen®, Mead-Johnson Nutritionals, Italy) resulted in symptomatic improvement, and the reintroduction of cow's milk after 4 wk caused symptoms to reappear. An open food challenge was performed in 5 of 8 subjects during hospitalization in consultation with a pediatric allergy specialist, and in 3 of 8 patients after discharge, in a tertiary Center of Pediatric Gastroenterology and Food Allergy.

Seven patients were classified as having intestinal infection according to the results of microbiological analysis. The microorganisms responsible for diarrhea are reported in Table 1. One patient initially classified as having gastrointestinal infection had a familial history of immunodeficiency and a clinical course characterized by growth delay, recurrent opportunistic infections, lymphopenia, associated with defective cellular and humoral immune responses. In this case, molecular analysis

Table 1 Etiology of diarrhea in the study population

	Acute	Chronic	Overall
Number of patients	36	3	39
Unknown etiology	10	-	10
Cow's milk allergy	8	-	8
Infections	7	-	7
Rotavirus	4	-	
Salmonella paratyphi	1	-	
Shigella flexneri	1	-	
Enterotoxigenic *Escherichia coli*	1	-	
Antibiotic-associated diarrhea	5	-	5
Neonatal withdrawal syndrome	2	-	2
Congenital diarrheal disorders	-	2	2
Glucose-galactose malabsorption	-	1	1
Congenital chloride diarrhea	-	1	1
Parenteral diarrhea			
Urinary tract infection	1	-	1
Hirschsprung's disease	1	-	1
Cystic fibrosis	-	1	1
Immunodeficiency			
Adenosine deaminase deficiency	1	-	1
Metabolic disorders			
Urea cycle defect	1	-	1

confirmed the clinical diagnosis of adenosine deaminase deficiency (OMIM 608958)[12].

Five babies presented with antibiotic-associated diarrhea. The microbiological analysis, including the search for *Clostridium difficile*, was negative in all these subjects.

According to clinical findings and the results of molecular analysis, two patients received a diagnosis of congenital diarrheal disorders (OMIM 251850): one with glucose-galactose malabsorption (OMIM 182380); and one with congenital chloride diarrhea (OMIM 214700)[13].

Neonatal withdrawal syndrome-induced diarrhea was reported in 2 subjects from mothers with a history of drug abuse (heroin and methadone) during pregnancy[14]. One case of Hirschsprung's disease was diagnosed according to the clinical history (no passage of meconium in the first 72 h of life, bloating of the abdomen) and the results of diagnostic tests, including rectal manometry, barium enema, and rectal biopsy[15]. This patient had diarrhea as a consequence of severe enterocolitis requiring broad-spectrum antibiotic therapy. Parenteral diarrhea induced by extra-intestinal infection caused by *Klebsiella pneumonia*-induced urinary infection was reported in one full-term baby during the first week of life. In this patient, diarrhea started before antibiotic treatment, all microbiological evaluations on stools were negative, and diarrhea improved rapidly when the urinary infection disappeared. For one subject with a familial history of cystic fibrosis (CF), intrauterine growth retardation, and chronic diarrhea, a final diagnosis of CF was achieved by a sweat test and was confirmed by the identification of a CFTR ΔF508 mutation, at the age of 4 mo[16]. A urea defect cycle was diagnosed in one patient by the presence of diarrhea together with metabolic acidosis, hyperammoniemia and protein load intolerance[17].

The main anamnestic and demographic characteristics of the neonates with diarrhea are shown in Table 2. Symptoms associated with diarrhea are reported in Table 3. The

Table 2 Main demographic and anamnestic data of the study population *n* (%)

	Acute	Chronic
Number of patients	36	3
Demographic data		
Male	15 (41.7)	1 (33.3)
Birth weight (g)	2504 ± 778	2560 ± 664
Gestational age (wk)	36.4 ± 3.8	37.7 ± 1.5
Age at diarrhea onset (d)	8.3 ± 6.6	5.7 ± 4.4
Age at hospitalization[a] (d)	6.2 ± 8.5	19.7 ± 10.1
Anamnestic data		
Intrauterine growth restriction	6 (16.7)	1 (33.3)
Polyhydramnios[a]	1 (2.8)	2 (66.7)
Premature birth	12 (33.3)	1 (33.3)
Familial for chronic diarrhea[a]	1 (2.8)	2 (66.7)
Newborns at high risk of developing allergy	12 (33.3)	-
Exclusively fed breast milk	3 (8.33)	-

[a]$P < 0.05$. Data are expressed as mean ± SD when not specified.

Table 3 Main associated symptoms in the study population *n* (%)

	Acute	Chronic
Number of patients	36	3
Gastrointestinal		
Vomiting	18 (50.0)	1 (33.3)
Mucus in the stools	13 (36.1)	1 (33.3)
Blood in the stools	9 (25.0)	-
Abdominal distension	24 (66.7)	2 (66.7)
Systemic		
Fever	4 (11.1)	-
Hypovolemia[a]	1 (2.8)	1 (33.3)
Eczema	7 (19.4)	-
Seizures[a]	3 (8.3)	1 (33.3)

[a]$P < 0.05$.

neonates affected by diarrhea of unknown origin showed a birth weight significantly lower (1920 g, IQR 1418 g) than subjects with an established etiologic diagnosis (2870 g, IQR 705 g) ($P < 0.040$). The mean number of bowel movements per day was 7.4 (95% CI: 7.0-7.8) without differences between acute and chronic diarrhea. The mean number of daily bowel movements was not influenced by etiology. The mean duration of diarrhea was similar for full term (9.08 d, 95% CI: 4.7-13.4) and preterm neonates (4.7 d, 95% CI: 3.9-5.6, $P = 0.953$). Linear regression analysis using a stepwise method demonstrated that diarrhea duration had a negative relationship with age at symptom onset (B -0.80; Beta -0.48; $P = 0.005$). In contrast, birth weight, sex, gestational age, antibiotic use, severity of dehydration, rehydration or re-feeding strategies, and consumption of breast milk did not correlate with duration of diarrhea.

Data on dehydration and rehydration strategies are reported in Table 4. As a re-feeding strategy in patients with acute diarrhea, an extensive casein hydrolyzed formula (Nutramigen®, Mead-Johnson Nutritionals, Italy) was administered to all subjects with a suspected diagnosis of CMA, and in only 6 of 31 of the remaining subjects (19.3%). Nine of 39 subjects with diarrhea were fed breast milk, 3 received breast milk exclusively at diarrhea

Table 4 Severity of dehydration, modalities of rehydration and clinical outcomes *n* (%)

	Acute	Chronic
Number of patients	36	3
Degree of dehydration		
Severe dehydration[a]	5 (13.9)	2 (66.7)
Rehydration modalities		
Exclusively oral rehydration	18 (50.0)	1 (33.3)
Exclusively parenteral rehydration	2 (5.6)	1 (33.3)
Oral plus parenteral rehydration	16 (44.4)	1 (33.3)
Clinical outcomes		
Duration of diarrhea[a] (d)	5.7 ± 2.5	44.3 ± 6.7
Electrolyte abnormalities	8 (22.2)	2 (66.7)
Deaths	2 (5.5)	1 (33.3)

[a]$P < 0.05$. Data are expressed as mean ± SD when not specified.

onset. After diarrhea onset, breast milk was continued in 3 subjects, temporally withdrawn (24 h) in 2, and definitively suspended in 4 of 9 newborn.

The clinical outcomes of diarrhea are reported in Table 4. Three deaths were reported among the 39 subjects (7.7%). Patients affected by adenosine deaminase deficiency, CF, and Hirschsprung's disease died due to complications related to fatal systemic infections at 1, 12 and 7 mo of life, respectively.

DISCUSSION

This is the first systematic study describing diarrhea in patients hospitalized in the NICU in an industrialized country and outside outbreak conditions. The results of our investigation showed that, in this particular setting, diarrhea is a relatively uncommon but insidious condition underlying a broad spectrum of illnesses. The list of diseases and mechanisms responsible for diarrhea in neonates is large and the number of possible etiologies is higher when compared to older pediatric patients[7,13,18,19]. In recent years, new diseases have been described (i.e. enteric anendocrinosis), and more accurate knowledge about neonatal enteropathies has been obtained[5,20]. The pathophysiology of these conditions is extremely variable and requires a complex diagnostic work up in order to rapidly adopt adequate therapy. We believe that all these steps should be performed as a result of tight collaboration between the neonatologist, pediatric gastroenterologist, immunologist, geneticist, and nutritionist. The most common etiology identified in our patients was food allergy (FA). Recently, the International Study of Asthma and Allergies in Childhood reported an increase in allergic disease prevalence occurring in the younger pediatric age group[21]. 20.5% of neonates in our population had FA-related diarrhea, and none were adherent to the recommendations of the AAP for primary prevention of FA[8]. These data highlight the importance of a high index of suspicion for FA in neonates presenting with diarrhea, and the application of preventive strategies[8,22,23]. The most frequent infective etiology was *Rotavirus,* which represents the leading cause of infectious diarrhea in childhood[24]. All patients acquired *Rotavirus* infection during hospitalization. Considering that *Rotavirus* is implicated in up to 50% of nosocomial pediatric diarrheal episodes, our data further support the importance of accurate surveillance against the spread of infection in the NICU[24].

In this study we reported the lack of a univocal rehydration and re-feeding strategy for hospitalized newborn with diarrhea, indicating the necessity for further prospective studies to optimize therapeutic approaches. The vast majority of newborn patients with diarrhea were rehydrated by the enteral route and received enteral feeding within 4-6 h, without complications. Despite the fact that it is difficult to assess the efficacy of rehydration and re-feeding strategies in patients with such different disorders, our results suggest that the therapeutic strategies for diarrhea which are commonly adopted in older subjects, could also be successfully used in newborn hospitalized in the NICU. This is of particular importance because the available guidelines for diarrhea management are mainly focused on older pediatric patients[7,25,26].

To conclude, despite the limits derived from the retrospective design, our study showed that neonatal diarrheal diseases are challenging clinical conditions due to their heterogeneous etiologies and possible severe outcomes. We believe that this study will help neonatologists to prevent diarrhea from becoming a severe clinical condition, and to recognize and correctly manage rare chronic cases who need the assistance of a specialized team dedicated to their long-term treatment. Specific guidelines for the management of diarrheal disorders in neonates are advocated.

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COMMENTS

Background

Diarrhea represents a major condition responsible for pediatric mortality worldwide. The onset of neonatal diarrhea may rapidly lead to life threatening dehydration and malnutrition. Thus, early diagnosis and timely treatment are both crucial in the management of diarrhea in neonates. Clinical and epidemiologic studies defining severity and etiology are needed in order to improve diagnostic and therapeutic approaches for the management of neonatal diarrhea.

Research frontiers

This study showed that neonatal diarrheal diseases are challenging clinical conditions due to their heterogeneous etiology and possible severe outcomes. The list of diseases and mechanisms responsible for diarrhea in neonates is large and the number of possible etiologies is higher compared with older pediatric patients. Specific guidelines for the management of diarrheal disorders in neonates are advocated.

Innovations and breakthroughs

This is the first systematic study describing diarrhea in patients hospitalized in the neonatal intensive care unit in an industrialized country and outside outbreak conditions. The results of the authors' investigation showed that, in this particular setting, diarrhea is a relatively uncommon but insidious condition underlying a broad spectrum of illnesses.

Applications

This research opens the way for new investigations in the area of diarrheal diseases with neonatal onset. The authors believe that these studies will help neonatologists to prevent diarrhea from becoming a severe clinical condition, and to recognize and correctly manage rare chronic cases who need the assistance of a specialized team dedicated to their long-term treatment.

Terminology

A neonatal intensive care unit, usually abbreviated to NICU, is a unit of a hospital specializing in the care of ill or premature newborn infants.

Peer review

This well-written informative and innovative manuscript convincingly shows that neonatal diarrheal diseases are challenging clinical conditions because of the heterogeneous etiology and possible severe outcomes. This study will help neonatologists to prevent diarrhea from becoming a severe clinical condition.

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BRIEF ARTICLE

Fields of applications, diagnostic yields and findings of OMOM capsule endoscopy in 2400 Chinese patients

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Abstract

AIM: To retrospectively analyze the fields of application, diagnostic yields and findings of OMOM capsule endoscopy in Chinese patients.

METHODS: A database including 2400 Chinese patients who received OMOM capsule endoscopy in 27 endoscopy centers in China was retrieved from the Jianshan Science and Technology Ltd. OMOM capsule endoscopy database. The patient's age, gender, fields of application, the potentially relevant findings, pyloric transit time (PTT), small bowel transit time (SBTT), and complete small-bowel examination rate (CSER) were recorded and analyzed.

RESULTS: Two thousand four hundred patients aged 9-91 years (mean, 49 years), of whom 1510 were males (62.9%), underwent 2400 OMOM capsule endoscopy procedures. One thousand two hundred and thirty two (51.3%) were referred with obscure gastrointestinal bleeding (OGIB), 642 (26.8%) with abdominal pain, and 223 (9.3%) with chronic diarrhea. The overall diagnostic yield was 47.7% (1144/2400). The diagnostic yield of OMOM capsule endoscopy in OGIB subgroup was much higher than in the non-OGIB subgroup (62.4% *vs* 32.1%, $P < 0.001$). The most common findings of the small bowel in Chinese patients with OGIB were arteriovenous malformation (28.1%) and tumors (18.9%). There was no significant difference in the diagnostic yield between the male and female patients with OGIB. However, the diagnostic yield in patients aged more than 60 was higher than in patients aged less than 60 (69.8% *vs* 58.9%, $P < 0.001$). The median PTT was 41 min (range: 1-544 min) and the mean SBTT was 247.2 ± 88.9 min. The overall CSER was 86.8%.

CONCLUSION: The OMOM capsule endoscopy is a valuable tool for small bowel evaluation with good overall diagnostic yield and CSER.

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Key words: OMOM capsule endoscopy; Obscure gastrointestinal bleeding; Small bowel

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INTRODUCTION

Examination of the small bowel represented a challenge for physicians due to its length and inaccessibility before the capsule endoscope and double balloon enteroscope

were introduced. On the other hand, radiologic techniques are relatively insensitive for diminutive, flat, infiltrative or inflammatory lesions of the small bowel. The capsule endoscope has been developed for direct and complete examination of the small bowel in a safe, non-invasive and well-tolerated manner and it has become the main method in the diagnosis of suspected diseases of the small bowel[1,2]. Today there are several different types of capsule endoscope systems in use. The M2A or PillCam capsule endoscope (Given Imaging, Israel) is the first wireless endoscopy system, which was approved by the Food and Drug Administration in August 2001. It has been also the most widely used capsule endoscope system with over 750 000 capsule ingestions worldwide[3-5]. Another less well known wireless capsule endoscope system, the OMOM capsule endoscope, developed by Jianshan Science and Technology (Group) Co., Ltd., Chongqing, China, has been widely used in China and some areas of Southeast Asian and European countries. It was approved by the State Food and Drug Administration of the People's Republic of China in March, 2004. About 60 000 OMOM capsules have been ingested since 2004[6]. However, there are few published studies with large sample size that summarize the experience of its clinical application. In this study we reported the fields of application, diagnostic yields, findings, pyloric transit time (PTT), small bowel transit time (SBTT), and complete small-bowel examination rate (CSER) of OMOM capsule endoscopy in 2400 Chinese patients.

MATERIALS AND METHODS

Database and patients

A database including 2400 cases was retrospectively retrieved from the Jianshan Science and Technology Ltd. OMOM capsule endoscopy database. The 2400 cases were performed at 27 endoscopy centers that used the OMOM capsule system in China.

Obscure gastrointestinal bleeding (OGIB) is defined as documented bleeding from the gastrointestinal tract with no etiology identified after upper endoscopy and colonoscopy. Each patient's age, gender, filed date of application, the potentially relevant findings, PTT (defined as the recorded time of the first image of duodenum), and SBTT (defined as the time from the first duodenal image to the time of the first cecal image for patients in whom the capsule reached the cecum and the time from the first duodenal image to the time of the last small-bowel image for patients in whom the capsule did not reach the cecum) were recorded and analyzed. Complete small-bowel examination was considered when the cecum was visualized during capsule endoscopy. The CSER was defined as the percentage of patients with complete small-bowel examination.

The OMOM imaging system

The OMOM capsule endoscopy system consists of four main parts: a smart capsule, an image recorder jacket, a portable real-time monitor, and a computer workstation. The OMOM capsule has an outer diameter of 13 mm and length of 27.9 mm and it weighs 6 g. Pictures are generally taken at a rate of 2 frames/s, though the rate can be changed during the study, a unique feature of this capsule endoscope system. There are 14 receiver elements placed close to the surface of the abdomen and waist in the recorder jacket. The duration of the battery of the OMOM capsule is about 8 h, similar to the PillCam small bowel capsule. The OMOM capsule endoscope transmits the acquired images *via* a digital radio frequency communication channel to the recorder jacket. A real-time monitor allows the endoscopist to view progression of the capsule and even to send various commands to the OMOM capsule through the workstation. The image format (VGA: 640 × 480; QVGA: 320 × 240), sampling frequency (capture rate of 2 fps, 1 fps or 0.5 fps), flash intensity (1-31 levels), exposure controlling (auto or manual), white balance (auto or manual), and the conditions of capsule (sleep or wakeup) can be adjusted through these commands. The workstation reading can be done at 1, 2, 4, 6, 8, or 15 frames per screen.

Statistical analysis

Quantitative data were summarized by mean ± SD or as medians with ranges, and analyzed by the *t*-test if they were normally distributed, or by the Wilcoxon rank-sum test if they were not normally distributed. Categorical data were presented as a frequency (percentage) and analyzed by a χ^2 test. SPSS 13.0 software for Windows (SPSS Inc., Chicago, Illinois, USA) was used for the statistical analyses. A P value of < 0.05 was considered statistically significant.

RESULTS

Age, gender and fields of application

Two thousand four hundred patients aged 9 to 91 years (mean, 49 years), of whom 1510 were males (62.9%), underwent 2400 OMOM capsule endoscopy procedures. 1232 (51.3%) cases were performed for OGIB, 642 (26.8%) for abdominal pain, and 223 (9.3%) for chronic diarrhea. Other fields of application included health examination (103, 4.3%), suspected Crohn's disease (52, 2.2%), and iron deficiency anemia (34, 1.4%) (Table 1).

Diagnostic yields

OMOM capsule endoscopies were negative in 1256 patients, and the overall diagnostic yield in the 2400 Chinese patients was 47.7%. OMOM capsule endoscopies were negative in 463 patients with OGIB and 793 patients with non-OGIB, respectively. The diagnostic yield of OMOM capsules in the OGIB subgroup was greater than in the non-OGIB subgroup (62.4% *vs* 32.1%, $P < 0.001$). In patients with abdominal pain and chronic diarrhea, the diagnostic yields were 39.4%, and 14.3%, respectively, and both were lower than that of patients with OGIB (both $P < 0.001$) (Table 2).

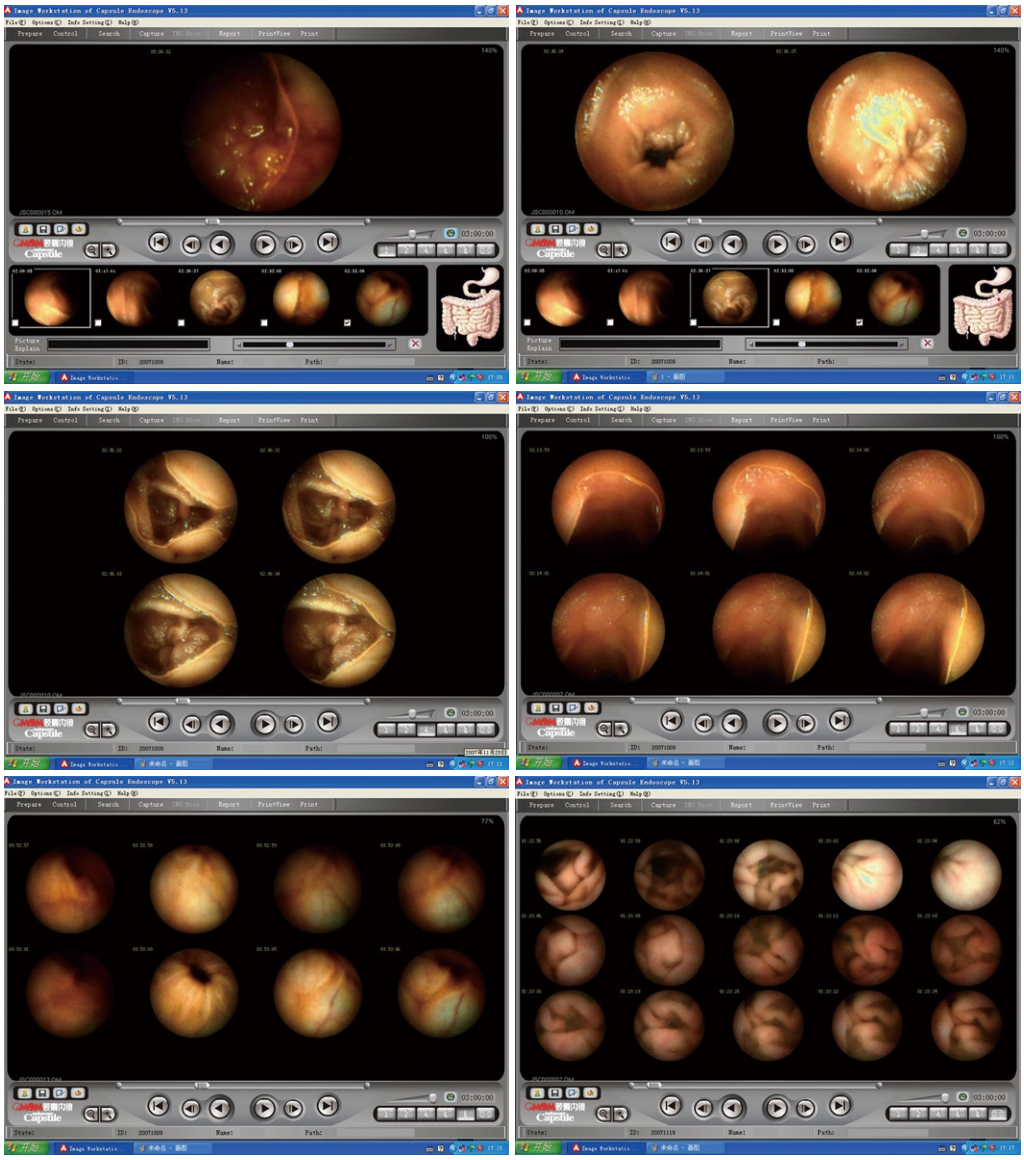


Figure 1 OMOM image workstation interfaces (1, 2, 4, 6, 8, or 15 frames per screen).

Table 1 Fields of application for OMOM capsule endoscopy in Chinese patients

Fields of application	*n* (%)
Obscure gastrointestinal bleeding	1232 (51.3)
Abdominal pain	642 (26.8)
Chronic diarrhea	223 (9.3)
Health examination	103 (4.3)
Suspected Crohn's disease	52 (2.2)
Iron deficiency anemia	34 (1.4)
Suspected tumors	25 (1.0)
Unexplained weight loss	19 (0.8)
Small intestinal polyposis	10 (0.4)
Ileus	10 (0.4)
Others	50 (2.0)
Total	2400 (100.0)

Findings in small bowel

Arteriovenous malformations were the most common finding, comprising 28.1% of 1144 patients with positive capsule endoscopy. The other common findings in the small bowel included tumor (216, 18.9%), polyp (119, 10.4%) and Crohn's disease (90, 7.9%). Small bowel parasites were also frequently found in 43 patients [*hookworm*, 26 (2.3%) and *lumbricoides*, 16 (1.4%)]. Celiac disease was rare in Chinese patients, accounting for only 0.6% (7) of the patients with positive capsule endoscopy (Figure 1).

In 1232 patients with OGIB, 769 potentially relevant lesions of the small bowel were seen on OMOM capsule endoscopy. The most common finding was arteriovenous malformations, which was seen in 313 patients (40.7%). The second most common finding in patients with OGIB was small intestinal tumors (201, 26.1%). The other common findings included small bowel ulcer (41, 5.3%), and polyps (24, 3.1%). Fifty seven patients (7.4%) had small intestinal active bleeding during the OMOM capsule endoscopy examinations. *Hookworm* and diverticulum were also causes for OGIB, and were seen in 26 (3.4%) and 18 (2.3%) patients. Interestingly,

Table 2 Small bowel findings on OMOM capsule endoscopy in 2400 Chinese patients *n* (%)

Findings	Overall	OGIB	Non-OGIB
Sum	2400 (100.0)	1232 (100.0)	1168 (100.0)
Negative	1256 (52.3)	463 (37.6)	793 (67.9)
Positive	1144 (47.7)	769 (62.4)	375 (32.1)
Arteriovenous malformations	322 (28.1)	313 (40.7)	9 (2.4)
Tumor	216 (18.9)	201 (26.1)	15 (4.0)
Polyp	119 (10.4)	24 (3.1)	95 (25.3)
Crohn's disease	90 (7.9)	23 (3.0)	67 (17.9)
Ulcer	89 (7.8)	41 (5.3)	48 (12.8)
Mucosal erosion	82 (7.2)	19 (2.5)	63 (16.8)
Duodenal ulcer	60 (5.2)	38 (4.9)	22 (5.9)
Active bleeding	59 (5.2)	57 (7.4)	2 (0.5)
Parasite	43 (3.8)	31 (4.0)	12 (3.2)
Hookworm	26 (2.3)	26 (3.4)	0 (0.0)
Lumbricoides	16 (1.4)	4 (0.5)	12 (3.2)
Strobilus	1 (0.1)	1 (0.1)	0 (0.0)
Lymphanglectasia	23 (2.0)	0 (0.0)	23 (6.1)
Diverticulum	21 (1.8)	18 (2.3)	3 (0.8)
Celiac diseases	7 (0.6)	0 (0.0)	7 (1.9)
Others	13 (1.1)	4 (0.5)	9 (2.4)

OGIB: Obscure gastrointestinal bleeding.

Table 3 Gender and age differences of small bowel findings on OMOM capsule endoscopy in 1232 Chinese patients with obscure gastrointestinal bleeding *n* (%)

Findings	Male	Female	Age ⩽ 60 yr	Age > 60 yr
Sum	731 (100.0)	501 (100.0)	838 (100.0)	394 (100.0)
Negative	277 (37.9)	186 (37.1)	344 (41.1)	119 (30.2)
Positive	454 (62.1)	315 (62.9)	494 (58.9)	275 (69.8)[b]
Arteriovenous malformations	189 (41.6)	124 (39.4)	206 (41.7)	107 (38.9)
Tumor	117 (25.8)	84 (26.7)	125 (25.3)	76 (27.6)
Polyp	14 (3.1)	10 (3.2)	16 (3.2)	8 (2.9)
Crohn's disease	17 (3.7)	6 (1.9)	16 (3.2)	7 (2.5)
Ulcer	28 (6.2)	13 (4.1)	33 (6.7)	8 (2.9)
Mucosal erosion	9 (2.0)	10 (3.2)	14 (2.8)	5 (1.8)
Duodenal ulcer	24 (5.3)	14 (4.4)	25 (5.1)	13 (4.7)
Active bleeding	28 (6.2)	29 (9.2)	25 (5.1)	32 (11.6)
Parasite	15 (3.3)	16 (5.1)	18 (3.6)	13 (4.7)
Hookworm	13 (2.9)	13 (4.1)	14 (2.8)	12 (4.4)
Lumbricoides	1 (0.2)	3 (1.0)	3 (0.6)	1 (0.4)
Strobilus	1 (0.2)	0 (0.0)	1 (0.2)	0 (0.0)
Diverticulum	10 (2.2)	8 (2.5)	12 (2.4)	6 (2.2)
Others	3 (0.7)	1 (0.3)	4 (0.8)	0 (0.0)

[b]$P < 0.001$, diagnostic yield, age > 60 years *vs* age ⩽ 60 years.

duodenal ulcers were found in 38 (4.9%) patients with OGIB by OMOM capsule endoscopy. There was no significantly difference in the diagnostic yield between male and female patients with OGIB. However, the diagnostic yield in patients aged more than 60 was higher than patients aged less than 60 (69.8% *vs* 58.9%, $P < 0.001$). The proportions of arteriovenous malformations and small bowel tumors were not significantly different between the male and female patients, and the young and old patients (Table 3).

In 1168 patients with non-OGIB, only 375 lesions of small bowel were found (32.1%). The most common finding was polyps (95, 25.3%). Other common findings in the small bowel included Crohn's disease (67, 17.9%), mucosal erosions (63, 16.8%), and ulcer (48, 12.8%).

Table 4 Small bowel findings on OMOM capsule endoscopy in Chinese patients with abdominal pain and chronic diarrhea *n* (%)

	Abdominal pain subgroup	Chronic diarrhea subgroup
Sum	642 (100.0)	223 (100.0)
Negative	389 (60.6)	191 (85.7)
Positive	253 (39.4)	32 (14.3)
Polyp	66 (26.1)	15 (46.9)
Mucosal erosion	60 (23.7)	0 (0.0)
Ulcer	39 (15.4)	1 (3.1)
Crohn's disease	22 (8.7)	4 (12.5)
Lymphanglectasia	22 (8.7)	1 (3.1)
Duodenal ulcer	18 (7.1)	0 (0.0)
Lumbricoides	10 (4.0)	1 (3.1)
Tumor	4 (1.6)	1 (3.1)
Diverticulum	1 (0.4)	0 (0.0)
Celiac diseases	1 (0.4)	6 (18.8)
Others	10 (4.0)	3 (9.4)

In 642 patients with abdominal pain, 253 small intestinal lesions were found. Polyps, mucosal erosions, and ulcers were the most common findings, and were seen in 66, 60, and 39 patients, respectively. Eighteen patients with duodenal ulcer were also diagnosed on capsule endoscopy. Only four small intestinal tumors were found in those patients. In 223 patients with chronic diarrhea, only 32 (14.3%) small intestinal lesions were found. Polyps, celiac disease, and Crohn's disease were seen in 15, 6, and 4 patients, respectively. In 103 subjects who received capsule endoscopy for the purpose of health examination, only 27 lesions were found (23.6%). Moreover, 15 lesions outside the small bowel were found. Small bowel polyp, diverticulum, mucosal erosion, and tumor were found in 7, 2, 2, and 1 subjects, respectively (Tables 4 and 5). Figure 2 showed some representative small bowel images captured by the OMOM capsule endoscope.

PTT, SBTT and CSER

PTT and SBTT were recorded in 1876 and 1420 patients, respectively. The SBTT was normal distributed, while PTT was not. The median PTT was 41 min (ranging 1-544 min). The mean SBTT of the 1420 patients was 247.2 ± 88.9 min, ranging from 27 to 720 min. There was no statistically significant difference between the male and female patients in the median PTT [40 (1-544) min *vs* 41.5 (2-465) min, $P = 0.155$] and mean SBTT (246.6 ± 89.7 min *vs* 248.3 ± 87.5 min, $P = 0.720$). In the patients aged over 60, the median PTT was shorter, while mean SBTT was longer than patients aged less than 60 [32.5 (2-474) min *vs* 43.0 (1-544) min, $P = 0.004$; 258.9 ± 87.2 min *vs* 245.1 ± 89.1 min, $P = 0.034$]. PTT was shorter in patients with OGIB [40 (1-474) min *vs* 43 (1-544) min, $P < 0.001$], while SBTT was a little longer (252.9 ± 92.5 min *vs* 241.3 ± 84.8 min, $P = 0.014$) than patients without OGIB.

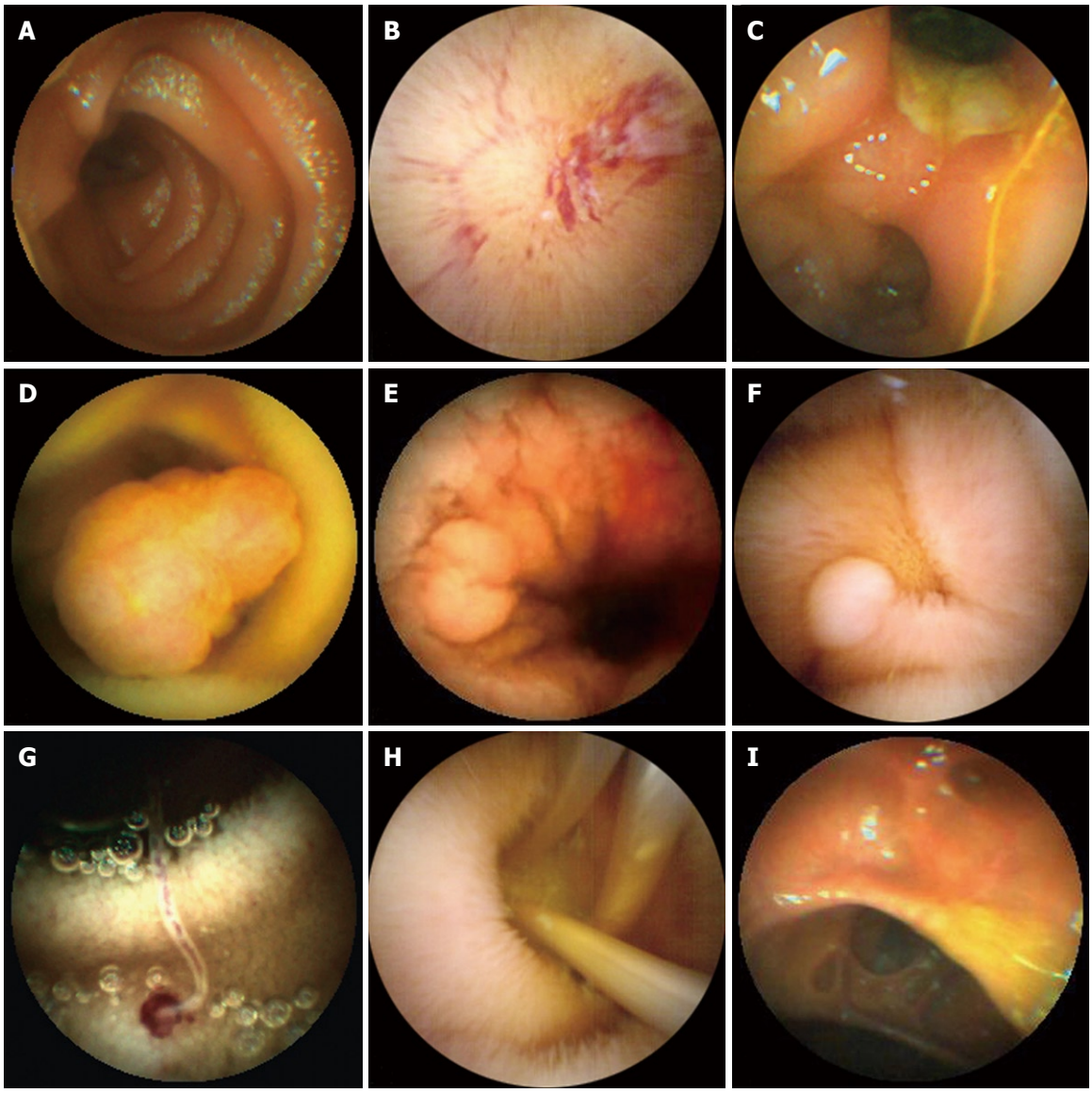


Figure 2 Small bowel images captured by the OMOM capsule endoscope. A: Normal small bowel mucosa; B: Angiectasias; C: Ulcer; D: Tumor; E: Crohn's disease; F: Polyp; G: *Hookworm*; H: *Lumbricoides*; I: Multiple diverticulum.

Table 5 Findings on OMOM capsule endoscopy in subjects for health examination *n* (%)

Findings	*n* (%)
Sum	103 (100.0)
Negative	76 (73.8)
Positive	27 (26.2)
Small intestinal polyp	7 (25.9)
Colonic polyp	4 (14.8)
Reflux esophagitis	3 (11.1)
Diverticula of colon	2 (7.4)
Duodenal ulcer	2 (7.4)
Small intestinal diverticula	2 (7.4)
Small intestinal mucosal erosion	2 (7.4)
Colonic ulcer	1 (3.7)
Duodenal diverticulum	1 (3.7)
Esophagopolypus	1 (3.7)
Gastric polyp	1 (3.7)
Small intestinal tumor	1 (3.7)

Two thousand and eighty two capsules reached cecums, and 318 capsules failed to reach cecums during the entire recording procedure. The overall CSER was 86.8%. In 318 patients with incomplete small-bowel examination, capsule endoscopies were negative in 140 patients. Crohn's disease (n = 41, 23.0%) and small bowel tumor (n = 40, 22.5%) were frequently found in the remaining 178 patients. Other findings included arteriovenous malformation and, ulcer. CSER was not significantly different between males (1321/1510, 87.5%) and females (761/890, 85.5%) (P = 0.171). CSER in patients aged over 60 was significantly lower than patients aged less than 60 (81.1% *vs* 87.9%, $P < 0.001$).

DISCUSSION

This was the first large report on a new wireless capsule

Table 6 The comparison of OMOM and PillCam SB capsule endoscopy

Technical specifications	OMOM	PillCam SB
Length (mm)	27.9	26
Diameter (mm)	13	11
Weight (g)	≤ 6	3.4
Frame rate (frames/s)	0.5/1/2 (adjustable)	2 (fixed)
Image sensor	CCD	CMOS
Shutter speed (s)	1/200	1/30
Image resolution (mm)	0.05	0.1
Pixel	300 000	80 000
Field of view (°)	140 ± 10	140
Illumination	6 white LEDs	6 white LEDs
Antennas (body leads)	14	8
Real-time (RT) view	RT monitoring	RT viewer
Recording time (h)	8-16	8
Capsule-workstation duplex communication	Yes	No
Cost, $ (in China)	400-800	1000-1200

endoscopy system, OMOM, developed by Jianshan Science and Technology Ltd., Chongqing, China. Our study has showed that OMOM capsule endoscopy is a valuable tool for small bowel evaluation with good overall diagnostic yield and CSER. The diagnostic yield in the OGIB subgroup was much higher than in the non-OGIB subgroup. Arteriovenous malformations and tumors of the small bowel were the most common causes in Chinese patients with OGIB.

The OMOM capsule is widely used in China and some areas of Asian and European countries. The OMOM capsule is slightly larger and heavier than the Given Imaging PillCam small bowel capsule. The duration of the battery of the OMOM capsule is about 8 h, similar to the PillCam small bowel capsule. Two unique features of the OMOM capsule system are real-time viewing and workstation-to-capsule command controlling capabilities[7]. However, there has been no reported comparison in the literature of the diagnostic yields of the two different capsule endoscope systems. The details and comparison with the available other capsule (i.e. PillCam), including the cost comparision, are shown in Table 6.

The most common applications for capsule endoscopy include evaluation for OGIB - including iron deficiency anemia, suspected Crohn's disease, suspected small intestinal tumors and surveillance in patients with polyposis syndromes, suspected or refractory malabsorptive syndromes (e.g. celiac disease)[3]. There is not yet consensus on indicated applications of capsule endoscopy from the Chinese Society of Digestive Endoscopy. In this study, though the most common indication for OMOM capsule endoscopy in Chinese patients was OGIB, many patients with abdominal pain and chronic diarrhea, even some healthy individuals, underwent OMOM capsule endoscopy. The study by Fry *et al*[8] suggested that capsule endoscopy had a low yield for evaluation of abdominal pain or chronic diarrhea and cannot be recommended as a first-line test without further study. Our study also found that the diagnostic yields by OMOM capsules in those patients were very low. Though the yields were a little higher than previous studies[8,9], it is unknown whether the findings of the small bowel in those patients were the potential relevant causes of abdominal pain or chronic diarrhea. Moreover, in the 103 patients who underwent health examination by OMOM capsule endoscopy, only 12 lesions of the small bowel were found. Strict patient selection on the basis of such symptoms or signs was the key to increasing the yield of capsule endoscopy. Small bowel capsule endoscopy is not recommended as a tool for health examination.

The yield of capsule endoscopy in OGIB in published reports ranges from 30% to 92%, depending on the definition of positive findings and the type of bleeding investigated[10]. In our study with more than 2000 cases that underwent OMOM capsule endoscopy, the data showed that overall yield of the OMOM capsule was nearly 50% in this patient group. This yield was similar to previous studies on PillCam small bowel capsule endoscopy[11]. The yield of the OMOM capsule endoscope in the OGIB subgroup was 62.4%, and was comparable with other published series using PillCam small bowel capsule endoscopy[12-15]. However, not all endoscopy centers in China had push enteroscopy and the database did not include the information on all investigations before capsule endoscopy. We also found the yield in the OGIB subgroup was greater than that in the non-OGIB subgroup, again suggesting that OGIB is the most important indication for capsule endoscopy and the OMOM capsule system probably has similar performance characteristics as those of the PillCam capsule system. Furthermore, we found that there was no significantly difference in the diagnostic yield between male and female patients with OGIB. We also found that the diagnostic yield in patients aged more than 60 was higher than patients aged less than 60. The proportions of arteriovenous malformations and small bowel tumors were not significantly different between young and old patients, which was different with the previous results by Papadopoulos *et al*[16]. Papadopoulos *et al*[16] found that older patients had significantly less erosions and normal studies, but they had more angiodysplasias. Our study showed that, in Chinese patients, the causes of OGIB were not significantly different between male and female patients, and young and old patients.

It is reported that arteriovenous malformations of the small bowel comprise over 60% of the lesions found on capsule endoscopy[12]. However, the percentage of arteriovenous malformations in this study was much lower than previous studies[14,17]. The second most common finding in Chinese patients with OGIB was small bowel tumors. Another Chinese study on double balloon enteroscopy also showed that the most common abnormalities in Chinese patients with OGIB were small bowel tumors (39.1%) and angioectasia (30.4%)[18]. The data suggested that the spectrum of causes of small bowel bleeding might be different between Chinese people and

Western countries populations. Interestingly, *hookworm* was seen in 26 Chinese patients with OGIB, comprising 3.4% of the positive findings in patients with OGIB. We also found that there were only six published case reports on *hookworm*-induced small intestinal bleeding on capsule endoscopy to date[19-24], and four out of six articles were from Asia. *Hookworm* infestation rate in Asian patients is probably higher than Western patients, but to prove this, further larger study is needed.

The incidence of Crohn's disease in Chinese population is increasing[25]. A bibliometrics study also suggested that the incidence and prevalence rates of Crohn's disease were still lower than those in Western and other industrialized Asian countries, but these rates have been increasing rapidly[26]. In a prior study, capsule endoscopy yielded a diagnosis of Crohn's disease only in a very small percentage of the patients (0% to 4%)[27]. In the present study, 90 patients with Crohn's disease were diagnosed by OMOM capsule endoscopy, suggesting that this disease is probably no longer uncommon in Chinese people.

Celiac disease is a gluten-dependent enteropathy, characterized by chronic small bowel inflammation and mucosal atrophy. Celiac disease is a common medical condition with an estimated prevalence of 1:100 in the general Western population[28]. Abdominal pain, chronic diarrhea, growth failure and malabsorption are its typical clinical presentation[29]. Preliminary reports suggest that capsule endoscopy could represent an attractive and non-invasive diagnostic tool in patients with suspected celiac disease[30,31]. However, our study showed that the prevalence of celiac disease in the Chinese patients who underwent capsule endoscopy was very low (7/2400, 0.3%). Up till now, there is no study which has reported on the prevalence of celiac disease in a general Eastern population.

Although the PTT and SBTT were not recorded in all 2400 patients, we found those values were similar to previous studies used Given Imaging capsule endoscopy[32]. The overall CSER of OMOM capsule endoscopy was 86.8%, which was a little higher than Given Imaging capsule endoscopy (83.5%)[33]. Previous studies[34,35] have shown that the real-time viewer of the Olympus Endocapsule is very useful for determining a need for detecting gastric transit abnormalities and necessary intervention. The real-time viewer of OMOM capsule endoscopy may help to secure the passage of the capsule endoscope into the small bowel and hence increase the rate of complete small bowel examination. Many centers in China use the real-time viewer of the OMOM capsule endoscopy system and the CSER can be increased in some patients, but real-time viewing is sometime time-consuming, and some patients cannot wait for long in the endoscopy center.

Though the sample size was large, there were some limitations in our study. Firstly, many Chinese capsule endoscopists reported mucosal erosions of the small bowel, but further definitive categorization of such lesions was not clear. Secondly, the follow-up results of patients with positive findings by OMOM capsule were unknown; the positive findings of Crohn's disease, celiac disease and tumor were not confirmed by biopsy, and the diagnostic yield may be overestimated.

In conclusion, this was the largest report on clinical application of the OMOM capsule endoscope system in Chinese patients. OMOM capsule endoscopy is a valuable tool for small bowel evaluation with good overall diagnostic yield and CSER. The yield in OGIB subgroup was much higher than in the non-OGIB subgroup. Arteriovenous malformations and tumors of small bowel were the most common causes in Chinese patients with OGIB.

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COMMENTS

Background

The OMOM capsule endoscope, developed by Jianshan Science and Technology (Group) Co., Ltd., Chongqing, China, has been widely used in China and some areas of Southeast Asian and European countries. However, there are few published studies with large sample size that summarize the experience of its clinical application.

Research frontiers

The capsule endoscope has been developed for direct and complete examination of the small bowel in a safe, noninvasive and well-tolerated manner; and it has become the main method in the diagnosis of suspected diseases of the small bowel.

Innovations and breakthroughs

This was the first large report on a new wireless capsule endoscopy system, OMOM, developed by Jianshan Science and Technology Ltd., Chongqing, China. This study has showed that OMOM capsule endoscopy is a valuable tool for small bowel evaluation with good overall diagnostic yield and complete small-bowel examination rate (CSER). The diagnostic yield in the obscure gastrointestinal bleeding (OGIB) subgroup was much higher than in the non-OGIB subgroup. Arteriovenous malformations and tumors of the small bowel were the most common causes in Chinese patients with OGIB.

Applications

OMOM capsule endoscopy is a valuable tool for small bowel evaluation with good overall diagnostic yield and CSER.

Terminology

Pyloric transit time: the recorded time of the first image of duodenum; small bowel transit time: the time from the first duodenal image to the time of the first cecal image for patients in whom the capsule reached the cecum and the time from the first duodenal image to the time of the last small-bowel image for patients in whom the capsule did not reach the cecum; CSER: the percentage of patients with complete small-bowel examination; OGIB: documented bleeding from the gastrointestinal tract with no etiology identified after upper endoscopy and colonoscopy.

Peer review

It is a nice paper about the use of a locally manufactured capsule. The authors should mention that this is a retrospective study. A bit more of the details and a comparison with the available other capsules (Pillcam) should be made. A cost comparison is also important.

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BRIEF ARTICLE

Small intestinal lipomas: Diagnostic value of multi-slice CT enterography

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Abstract

AIM: To analyze the clinical and imaging features of the small intestinal lipomas and to evaluate the diagnostic value of multi-slice computed tomography (CT) enterography.

METHODS: Fourteen cases (one had two intestinal lesions) of surgically confirmed lipomas of the small intestine were retrospectively analyzed. The location, size, clinical and radiological aspects were discussed.

RESULTS: Twelve patients presented with abdominal pain, of whom three complained of paroxysmal colic. Melena or bloody stools was mentioned in five cases. One lesion was detected incidentally during routine physical examination. One lesion was found unexpectedly during the preoperational evaluation for cholecystitis. Examination of the abdomen revealed palpable masses in four cases. Precontrast CT scan showed round or oval well-defined hypo-intense intraluminal masses with the attenuation ranging from -130 HU to -60 HU. On contrast enhancement CT scan, no striking enhancement was seen.

CONCLUSION: The small intestinal lipomas are rare and difficult to diagnose merely based on clinical manifestations, while the characteristic features at small intestinal CT enterography can help establish reliable prospective diagnoses.

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Key words: Lipoma; Small bowel; Computed tomography; X-ray; Enterography

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INTRODUCTION

Primary small intestinal tumors are uncommon, which account for about 1% of all gastrointestinal tumors, and primary lipomas of the small intestine are rare[1-5]. Preoperative diagnosis is usually indeterminate only based on clinical manifestations due to the overlap of symptoms and signs with other intestinal tumors. Benign tumors in the small intestine do not have a characteristic computed tomography (CT) appearance and, in most instances,

cannot be differentiated from malignant lesions, but lipomas can be definitively diagnosed by the recognition of fat attenuation within the mass[1]. To the best of our knowledge, only case reports of small bowel lipoma were presented in the literature[1-4]. Herein we present 15 surgically confirmed small intestinal lipomas and analyze retrospectively their clinical, pathological and imaging findings, especially multi-slice CT enterography.

MATERIALS AND METHODS

General data

The institutional review board approved this retrospective study and waived the need to obtain patient consent. Fourteen patients (one had two intestinal lesions) treated at three hospitals (Sir Run Run Shaw Hospital; First Affiliated Hospital, Zhejiang University School of Medicine and Zhejiang Provincial People's Hospital, China) from May 1994 to June 2009 were enrolled. All patients (four women and ten men; age range, 19-82 years; mean age, 63.2 years) with pathologically confirmed lipoma underwent 16-detector row CT enterography before surgery.

Clinical manifestations

Melena or bloody stools was mentioned in five cases. Fecal occult blood test was positive in nine cases and negative in the other five cases. No significant abnormality was found in blood biochemical tests. Twelve patients presented with abdominal pain, three of them complained of paroxysmal colic and were subsequently found to have intestinal obstruction secondary to intussusception. They denied vomiting, fever, or chills. One lesion was detected incidentally during routine physical examination. One lesion was found unexpectedly during the preoperational investigation for cholecystitis. Physical examination of the abdomen revealed palpable masses in four cases.

RESULTS

Fourteen patients had confirmed small intestinal lipomas (Figures 1-3), including one who had two intestinal lesions. Four of the 15 tumors occurred in the duodenum, three in the jejunum, and eight in the ileum (Figures 1-3).

The size of the lesions ranged from 2 cm × 1.5 cm × 1.1 cm to 6.3 cm × 4.5 cm × 2 cm (mean, 3.9 cm × 2.8 cm × 2.3 cm). The tumors were round or oval (Figures 1-3) and three were pedunculated in shape.

Precontrast CT scan showed round or oval well-circumscribed hypo-intense intraluminal masses with the attenuation ranging from -130 HU to -60 HU (Figure 2). Eleven of the 15 tumors were homogeneous, while the remaining four were heterogeneous with several streak septa. No enhancement after intravenous contrast material administration (Figures 1-3) was found in all the lesions, except the streak septa which was enhanced minimally. Small intestinal CT enterography demonstrated the typical "target" appearance (Figure 1) as intestinal intussusception in three cases. Except one of the three patients with intussusception, all other 13 patients had a definitive preoperative diagnosis of lipomas by the CT enterography, which were confirmed by subsequent surgical pathology.

At laparotomy, a jejunojejunal or ileoileac intussusception (Figures 1 and 3) was present in three cases. One patient had two separated submucosal lipomas. All tumors were defined to originate from the submucosal layer. Grossly they had a clear margin and yellow section surface. Microscopically (Figure 1D), they were composed of mature adipose cells, nine tumors had overlying necrosis and ulceration, and four showed inflammatory fibrous hyperplasia with septa formation extending in the adipose tissue.

DISCUSSION

Tumors of small intestine account for only 1%-2% of all gastrointestinal tumors, and benign tumors account for approximately 30% of all small intestinal tumors[6]. Gastrointestinal stromal tumors are the most common symptomatic benign tumors of the small bowel, while the lipomas are rare benign tumors, representing 2.6% of non-malignant tumors of the intestinal tract[2].

Pathology

Lipomas are often single submucosal intraluminal masses, with intensely yellow color in the mucosa because of the underlying accumulated fat. They usually appear as a capsulated round or oval yellowish mass protruding into the intestinal lumen. Sometimes there is overlying ulceration of the mucosa[7-10].

Clinical manifestations

Small bowel lipomas tend to occur in the elderly, and the sixth to seventh decades of life are considered to be the most risky period[3,11,12]. Jejunum and ileum, especially the terminal ileum, are common locations. Eleven of the 15 lesions arose from jejunoileum and the mean age of the patients was 63.2 years in this study, which was consistent with those previously reported. Patients may be completely asymptomatic with small lipomas (< 1 cm), which are usually detected incidentally. Larger tumors may produce symptoms as follows.

Paroxysmal abdominal pain: About 75% patients present with abdominal pain in different degree. Twelve of the 14 patients in this study complained of abdominal pain and 3 suffered from severe colic due to intussusception. Tumor traction, peristalsis disorder, intestinal obstruction, tumor necrosis or secondary infection may result in abdominal pain while the intestinal obstruction due to lipomas is usually characterized clinically by recurrent severe abdominal pain with increased bowel sound and locally palpable round or oval masses. Lesions greater than 4cm in diameter have a greater likelihood of introducing bowel intussusception, and submucosal or subserosal lipomas also tend to cause intussusception[7,13-15]. Large subserosal tumors are prone to result in intestinal compression and volvulus.

Hemorrhage of digestive tract: Approximately 25% of

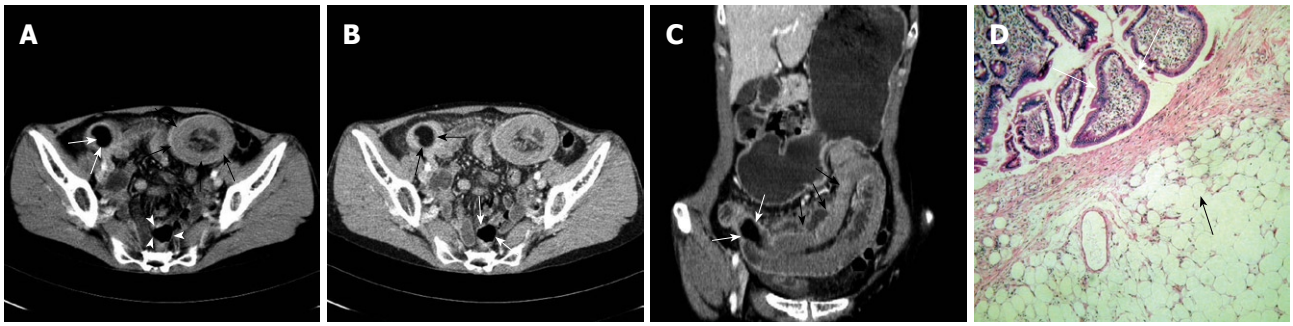


Figure 1 A lipoma of the ileum in a 56-year-old woman. A: Intussusception (black arrows) secondary to a lipoma. On transverse enhanced computed tomography (CT) scan, a well-defined hypo-attenuating mass (white arrows) is revealed with no enhancement, but it is difficult to differentiate the tumor from the intraluminal gas (arrowheads); B: The same level image with Figure 1A. It is easy to differentiate the lipoma (black arrows) from the intraluminal gas (white arrows) with regulated image windows; C: Small intestinal two-dimensional (2D) CT enterography shows ileoileac intussusception (white arrows) secondary to a lipoma (black arrows); D: Photomicrograph reveals the lipoma (black arrow) in the submucosal layer, the intestinal normal mucous membrane is also seen (white arrows) (HE, × 40).

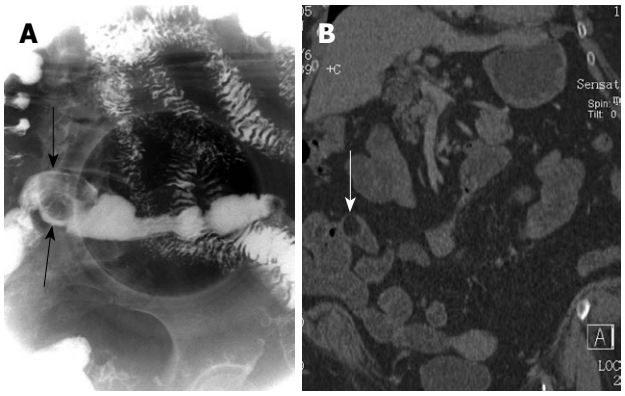


Figure 2 A lipoma of the ileum in a 70-year-old woman. A: Gastrointestinal barium examination shows an oval well-circumscribed intraluminal mass in the ileum (black arrows); B: Small intestinal 2D CT enterography reveals oval well-circumscribed hypo-intense (-89 HU) intraluminal mass (white arrow) in the ileum.



Figure 3 A lipoma of the ileum in a 59-year-old woman. Small intestinal 2D CT enterography showing intussusception secondary to a lipoma (white arrow).

patients with benign intestinal neoplasm have alimentary tract hemorrhage. Small bowel lipomas usually cause lower gastrointestinal hemorrhage, and lipomas arising from duodenum may cause upper gastrointestinal hemorrhage and symptoms like peptic ulcer. Some of the bleeding is intermittent, some is continuous in small amount or only presents as microscopically positive fecal occult blood. Melena or bloody stools was mentioned in five cases and fecal occult blood test was positive in nine cases in this study.

Abdominal masses: Large lipomas may be palpable as soft, lobulated and movable abdominal masses without tenderness. Of the 4 patients who had palpable masses in this study, three were ill-defined with tenderness, possibly due to the intestinal intussusception.

Other reported clinical symptoms include nausea, vomiting, constipation, diarrhea and abdominal distention. Pedunculated submucosal lipomas may ablate spontaneously and discharged from anus when there are volvulus and necrosis.

Diagnosis

Preoperative diagnosis of the small intestinal lipomas is mainly established by means of endoscopic and radiologic evaluations. The duodenal lipomas are endoscopically characterized by: (1) "tenting sign", implying that the mucosa can be pulled from the lesion with biopsy forceps on endoscopy; and (2) "cushion sign", implying spongy consistency of the lesion when indented with biopsy forceps and yellowish change of the mucosa from normal pink appearance for the compression of the vessels, representing the underlying lipoma[16].

The diagnosis of the jejunoileal lipomas mainly depends on the imaging modalities as follows.

The role of abdominal conventional radiographs for the evaluation of lipomas is limited. Mechanic intestinal obstruction or low density of the lipid is occasionally seen[17]. Abdominal X-ray films of two patients in our series revealed small bowel obstruction but failed to suggest the primary disease.

Double-contrast barium study, especially small bowel enema, may be helpful for the lipomas evaluation. The tumor commonly presents as round, oval or lobulated well-circumscribed intraluminal filling defect (Figure 2A), with so-called "squeeze sign" in which the shape of the mass changes as a consequence of peristalsis or manual pressure[3]. Some pedunculated tumors are movable and may change their locations in response to position or palpitation. There is also so-called "bull's-eye sign" signifying a niche formation in the radiolucent filling defect due to ulceration. The intestinal peristalsis remains normal and the mucosa is intact. However, barium study still has limitations in many aspects including: (1) overlap of the

intestinal loops, which makes unsatisfactory display and may result in misdiagnosis; (2) difficult differentiation from other tumors which also present as filling defects; (3) disability to detect small lipomas; and (4) contraindication in patients accompanying intestinal obstruction or intussusception.

Ultrasound may detect large lipomas of the small intestine as hyper- or hypo-echoic masses, or irregular sausage-shaped masses, of which the inner central part is hyper-echoic and the peripheral area is hypo-echoic representing intestinal intussusception[15,17]. However, the role of ultrasound is also limited greatly because of the noise interference caused by the intraluminal gas. Ultrasound examination of the two cases of our series both failed to establish a correct diagnosis.

Abdominal CT scans are considered to be the most definitive diagnostic measure of recognizing small bowel lipomas[1,3,11,15]. With the fast volume scan technique of multiple spiral CT, reformation in any dimensions is feasible, and the quality of the reformatted images is almost as same as the axial ones, which ensures a good overall observation. The finding of lipid attenuation (-100 to -50 HU) is vital to the diagnosis of a lipoma[17,18]. CT examination revealed lipid-attenuated (-130 to -60 HU) intraluminal masses in all patients of our series (Figure 2B), the location and range of the tumors and the accompanying intussusception were also displayed clearly and directly on the two-dimensional (2D) CT enterography (Figure 1C and Figure 3), which helped establish a correct prospective diagnosis. For the other misdiagnosed cases, postoperational recognization of the reformatted CT images with regulated windows still retrospectively showed the tumor existence. It is important to stress that adequate oral contrast media is necessary for filling the small bowel and optimal windows is also needed to avoid mistaking lipomas for intraluminal gas. Observation of the images reformatted in multiple dimensions is helpful in differentiation lipomas from invaginated mesenteric fat when there is an intestinal intussusception.

The role of MR imaging in the evaluation of small bowel lesions is limited due to the lack of effective intestinal contrast media and the presence of motion artifact caused by relatively long scanning time and intestinal peristalsis. With new MR machine, advanced fast scan techniques, fat-saturation imaging and gadolinium chelates application, the resolution of MR images has improved, the clinical and research application on intestinal diseases has increased over recent years, nevertheless there are still few reports in the literature about MR imaging of small bowel lipomas[19,20].

Clinical manifestation is not specific for this rare entity, imaging modalities are helpful for revealing small bowel lipomas, especially the characteristic features at multi-slice CT enterography can be used by the radiologists to establish a definitive diagnosis before operation for most cases. In the instances of secondary intestinal intussusception or obstruction, the primary lesion of a lipoma should be carefully searched out.

COMMENTS

Background

Lipomas are benign tumors and may arise from any anatomic location, subcutaneous tissue is the most common site, while lipomas of the alimentary tract especially of the small intestine are rare entities. According to the authors of this paper, only case reports of small bowel lipoma have been presented in the literature.

Research frontiers

Primary small bowel tumors are uncommon, which account for about 1% of all gastrointestinal tumors, and primary lipomas of the small intestine are rare. Preoperative diagnosis is usually indeterminate only based on clinical manifestations due to the overlap of symptoms and signs with other intestinal tumors. Benign tumors in the small intestine do not have a characteristic computed tomography (CT) appearance and, in most instances, cannot be differentiated from malignant lesions, but lipomas can be definitively diagnosed by the recognition of fat attenuation within the mass.

Innovations and breakthroughs

The authors presented 15 surgically confirmed small intestinal lipomas and analyzed retrospectively their clinical, pathological and imaging findings, especially multi-slice CT enterography.

Applications

Clinical manifestation is not specific for the small intestinal lipomas, imaging modalities are helpful for revealing small bowel lipomas, especially the multi-slice CT enterography which can be used by the radiologists to establish a definitive diagnosis before operation for most cases.

Terminology

Small bowel lipomas tend to occur in the elderly, and the sixth to seventh decades of life are considered to be the most risky period. Multi-slice CT enterography (MCTE): MCTE is a new non-invasive procedure that uses very fast CT scanning combined with liquid intake to obtain detailed images of the abdomen. CT enterography has several advantages over conventional bowel imaging: it shows the entire thickness of the bowel wall; it is able to image all of the long loops of the small intestine; and it can also evaluate surrounding mesentery and fat tissues. CT enterography has also become an important alternative to traditional fluoroscopy in the assessment of other small bowel disorders such as celiac sprue and small bowel neoplasms.

Peer review

The authors present 15 surgically confirmed small intestinal lipomas and analyze retrospectively their clinical, pathological and imaging findings.

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BRIEF ARTICLE

Microsurgical reconstruction of hepatic artery in A-A LDLT: 124 consecutive cases without HAT

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Abstract

AIM: To retrospectively investigate microsurgical hepatic artery (HA) reconstruction and management of hepatic thrombosis in adult-to-adult living donor liver transplantation (A-A LDLT).

METHODS: From January 2001 to September 2009, 182 recipients with end-stage liver disease underwent A-A LDLT. Ten of these patients received dual grafts. The 157 men and 25 women had an age range of 18 to 68 years (mean age, 42 years). Microsurgical techniques and running sutures with back-wall first techniques were performed in all arterial reconstructions under surgical loupes (3.5 ×) by a group of vascular surgeons. Intimal dissections were resolved by interposition of the great saphenous vein (GSV) between the donor right hepatic artery (RHA) and recipient common HA (3 cases) or abdominal aorta (AA) (2 cases), by interposition of cryopreserved iliac vessels between the donor RHA and recipient AA (2 cases).

RESULTS: In the 58 incipient patients in this series, hepatic arterial thrombosis (HAT) was encountered in 4 patients, and was not observed in 124 consecutive cases (total 192 grafts, major incidence, 2.08%). All cases of HAT were suspected by routine color Doppler ultrasonographic examination and confirmed by contrast-enhanced ultrasound and hepatic angiography. Of these cases of HAT, two occurred on the 1st and 7th d, respectively, following A-A LDLT, and were immediately revascularized with GSV between the graft and recipient AA. HAT in one patient occurred on the 46th postoperative day with no symptoms, and the remaining case of HAT occurred on the 3rd d following A-A LDLT, and was cured by thrombolytic therapy combined with an anticoagulant but died of multiorgan failure on the 36th d after A-A LDLT. No deaths were related to HAT.

CONCLUSION: Applying microsurgical techniques and selecting an appropriate anastomotic artery for HA reconstruction are crucial in reducing the high risk of HAT during A-A LDLT.

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Key words: Adult-to-adult living donor liver transplantation; Hepatic arterial thrombosis; Microsurgical reconstruction

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INTRODUCTION

In the face of a constant shortage of cadaveric livers and the increasing size of the transplantation waiting list, living donor liver transplantation (LDLT) has become an accepted therapy for many patients with end-stage liver disease. However, despite improvements in LDLT, vascular complications related to the small diameter of the vessels in the partial liver graft and complex arterial reconstruction remain an important cause of morbidity and mortality[1-3]. Hepatic artery thrombosis (HAT), which is the most common vascular complication after LDLT, can result in graft loss associated with septic hepatic infarction and bile duct ischemia[4,5]. The incidence of HAT after LDLT varies widely, with a reported frequency of 4%-25%[6-10].

Retransplantation used to be an option for the treatment of HAT[11]. However, retransplantation is limited by both organ availability and the patient's condition. Urgent revascularization with thrombectomy or revision of anastomosis with thrombectomy has been successful in some patients with an early diagnosis[8,9]. Color Doppler ultrasonography[12] and contrast-enhanced ultrasound (CEUS)[13] have provided an accurate noninvasive method for the detection of HAT before nonreversible ischemic damage of the graft occurs.

The application of microsurgical techniques in arterial reconstruction has helped overcome the high risk of HAT following liver transplantation, and the effectiveness of this method has been demonstrated in numerous reports[14-17]. Because of the short donor hepatic artery (HA) stump which is small in diameter and vessel size discrepancy between the graft and recipient hepatic arteries, reconstruction of hepatic arteries is a challenge to surgeons in LDLT.

To outline our experience in hepatic reconstruction and the management of HAT after adult-to-adult living donor liver transplantation (A-A LDLT), the clinical data of patients with end-stage liver disease who underwent A-A LDLT at our center, were retrospectively reviewed.

MATERIALS AND METHODS

Recipients

From January 2001 to September 2009, we performed 182 consecutive A-A LDLTs in adult recipients with a mean age of 42 years (157 men and 25 women, aged 18 to 69 years) suffering from end-stage liver disease. Informed consent from both donor and recipient and the approval of the Ethics Committee of West China Hospital of Sichuan University were obtained. Of these cases, 10 recipients underwent dual graft liver transplantation.

Table 1 Details of recipients and diagnostic indications (n = 182)

	n (%)
Female	25 (13.74)
Male	157 (86.26)
Age (yr)	
18-60	173 (95.05)
60-69	9 (4.95)
Diagnostic indications	
Liver cirrhosis	74 (40.66)
Hepatitis B	52 (28.57)
Hepatitis C	5 (2.75)
Cholestasis	5 (2.75)
Alcoholic	4 (2.20)
Others	8 (4.39)
Hepatic carcinoma	77 (42.31)
FIF	10 (5.49)
Acute-on-chronic hepatic failure	9 (4.94)
Budd-Chiari[19]	4 (2.20)
DI IBS	4 (2.20)
Post-trauma hepatic failure	1 (0.55)
Hepatic echinococcosis	2 (1.10)
Polycystic liver	1 (0.55)
MELD	
1-13	86 (47.25)
14-24	63 (34.62)
⩾ 25	33 (18.13)

FIF: Fulminant liver failure; DI IBS: Diffuse ischemic intrahepatic biliary stenosis; MELD: Model for end-stage liver disease.

In addition to dual graft A-A LDLT, 170 recipients received right grafts (right lobe without MHV, 160; right lobe with MHV, 10), 2 recipients received left grafts (extended left lateral segment, 1; left lobe without MHV, 1). The pre-transplant condition of the recipients was evaluated by the modified model for end-stage liver disease scores which has been documented to better predict the prognosis of patients[18]. The details of recipients and the diagnostic indications for A-A LDLT are summarized in Table 1. All these patients underwent emergency A-A LDLT.

Donors

There were 190 donors, including 110 men and 80 women, with an age range of 19-65 years (mean age, 35 years). Details of the dual donors are described in Table 2. The donors and recipients were blood group identical in 151 cases and compatible in 31 cases. All donors in this group voluntarily donated part of their liver following informed consent.

Preoperative evaluation of donors and recipients

Physical examination and retrospective analysis of both donors' and recipients' medical records were performed before surgery. Hepatitis, syphilis, HIV, Epstein-Barr virus, cytomegalovirus, and tuberculosis infection constituted ineligibility as a potential donor. We did not use routine HA angiography to study the tracks and variations of the HA, but used computed tomographic arteriography (CTA) instead. The volume of the total

Table 2 Details of dual donors

No.	Donor 1			Donor 2		
	Gender	Age (yr)	Graft	Gender	Age (yr)	Graft
1	F	34	LL	F	31	LL
2	F	56	RL	M	27	Cadaveric LL
3	F	35	RL	M	55	LLS
4	F	29	RL	M	29	Cadaveric LL
5	M	42	RL	F	29	LL
6	M	58	LL	F	34	RL
7[1]	M	45	RL	F	26	RL
8	F	34	RL	M	35	LL
9	F	20	RL	F	39	LLS
10[2]	F	28	RL	F	28	RL

[1,2]These donors donated their right lobes, respectively, the others donated right lobes without the middle hepatic vein. LL: Left lobe; RL: Right lobe; LLS: Left lateral segment.

liver and right lobe were evaluated and calculated with 3-dimensional computed tomography. Graft mass to recipient body weight ratio (GRWR) of 0.8%[20] or graft volume to recipient standard liver volume ratio of 40%[21] and donor remnant liver volume of 35%[22] were required for recipient and donor safety assurance.

Donor surgery and back table

The detailed surgical technique was as previously reported[23]. The partial graft liver was harvested without vascular flow occlusion and without graft manipulation to maintain graft viability. The HA and portal vein (PV) were individually exposed and carefully divided, during hilar dissection. In right grafts, the right hepatic artery (RHA) was identified and isolated to the right side of the hepatic duct. In left grafts, the proper hepatic artery (PHA) was isolated up to the bifurcation of the left hepatic artery (LHA) and the RHA. We preserved the length of the arteries as much as possible and cut the RHA or LHA with sharp scissors or a microvascular appliance to avoid injury to the arterial intima and intimal dissection.

All grafts were placed into a container filled with 4℃ University of Wisconsin (UW) solution, then removed to the back table. On the back table, we perfused the grafts from the PV with UW solution, rinsed the biliary tracts and flushed arterial ducts with heparin solution. All procedures mentioned below were performed by vascular surgeons. Plasty of the hepatic vein and PV was performed to make a single orifice for each. We anastomosed the great saphenous vein (GSV) from the recipient or cryopreserved vessels to the crassitude tributaries of the MHV (diameter, > 5 mm). We inspected the graft arteries to detect whether intimal injures and dissection existed. When the graft arterial stump was shorter than 1 cm, we anastomosed the inverse GSV from the recipient to the graft arterial stump to prolong the stump and interposed the GSV between the graft artery and recipient artery. The graft HA including the origins of multiple tiny arteries was reshaped to achieve a single orifice of adequate diameter at the back table.

Recipient surgery

The procedure was performed following the routine procedures of our hospital[24,25]. All recipient hepatic arteries were isolated toward their insertion sites into the hepatic parenchyma. After removal of the recipient liver, the stump of the recipient HA was meticulously preserved for as long as possible. The implanted graft was reperfused after reconstructing both the hepatic and PVs. Thereafter, one group of vascular surgeons performed all the microvascular reconstructions of the HA. Hepatic arterial reconstruction was performed using microvascular techniques with (3.5 ×) surgical loupes, after adoption of systemic anticoagulation (heparin, 62.5 U/kg, intravenous, 5 min before anastomosis). Low dose heparin did not cause any remarkable adverse effects in our study. We used running 8-0 or 9-0 monofilament polypropylene (Prolene; Ethicon Inc.) sutures for the HA anastomosis depending on the arterial diameter. During running sutures, the assistant vascular surgeon persistently and delicately stretched the stitch to ensure intimal eversion, and the vascular surgeon could see the arterial lumen after each stitch. According to the amount of inflow, stump location and matching of diameters, the most appropriate inflow artery was selected. In all cases, end-to-end vessel anastomosis was carried out between the recipient and graft HA, and the back wall-first microsurgical anastomosis technique which avoided vessel twisting during suturing was applied. In the case of a hepatic graft with multiple stumps, we tried to reconstruct all of the stumps. The dominant HA was reconstructed first. If there was sufficient back bleeding, the other arterial stumps were ligated. If there was only weak back bleeding, we always reconstructed the stumps. The patency of the arterial anastomosis was evaluated by intraoperative Doppler ultrasonography.

In dual graft patients, the RHA of the graft was anastomosed to the RHA of the recipient to reconstruct the RHA. Finally, the living or cadaveric left lateral segment was orthotopically implanted to the left lobe position of the recipient, followed sequentially by end-to-end anastomosis. The primary candidates of the recipient HAs for reconstruction were the RHA, LHA, and PHA. There were 192 anastomoses in all A-A LDLTs. Details of the recipient arteries are described in Table 3. The caliber difference between the graft artery and the recipient artery was resolved by cutting the smaller artery obliquely. Because of recipient HAs intimal dissection, these arteries were not suitable for HA reconstructions, and interposition bypass using GSV was performed between the donor RHA and recipient common hepatic artery (CHA) in 3 cases. Bypass was performed between the donor RHA and recipient abdominal aorta (AA) using GSV in 2 patients and cryopreserved cadaveric iliac vessels in 2 cases. The gastroduodenal artery was not ligated to increase the blood flow through the anastomosis. Administration of alprostadil (20 μg) to maintain artery patency was used in all cases after the completion of hepatic arterial reconstruction.

Table 3 Details of hepatic arteries (n = 192)

	n (%)
RHA	62 (32.29)
LHA	20 (10.42)
PHA	90 (46.88)
CHA	14 (7.29)
Aberrant RHA[1]	2 (1.04)
AA	4 (2.08)

[1]Aberrant right hepatic artery arising from superior mesenteric artery. Diameter of graft: mean (range), 1.91 (1.5-2.5) mm; diameter of recipient: mean (range), 2.12 (1.5-3.5) mm. RHA: Right hepatic artery; LHA: Left hepatic artery; PHA: Proper hepatic artery; CHA: Common hepatic artery; AA: Abdominal aorta.

Postoperative management and follow-up

All patients received immunosuppressive therapy including cyclosporine or tacrolimus. All patients underwent Doppler ultrasonography every 12 h during the first postoperative week and daily during the second postoperative week to confirm HA patency. The diagnosis of HAT after A-A LDLT was based on clinical presentation, color Doppler ultrasonography findings, and HA arteriography. If elevated hepatic enzymes, cholestasis, bile leakage, or high fever in the absence of acute rejection were detected, color Doppler ultrasonography, CTA, or conventional HA angiography was performed to establish the diagnosis in turn. If hepatic arterial inflow was not observed by color Doppler ultrasonography, CUES examination was performed in these cases[12].

RESULTS

Donor outcome

There were no donor deaths, and the hospitalization period ranged from 7 to 30 d (mean 10 d). Only 4 donors had the following complications: portal venous thrombosis in 1 case who underwent second thrombectomy and leakage was mended with a patch of GSV; transient chyle leakage in 1 case, which healed after symptomatic treatment; subphrenic effusion in 1 case which was cured by surgical drainage; and pleural effusion, healed after repeated thoracic cavity puncture.

Recipient outcome

In this series, HAT occurred in 4 cases of 192 grafts after A-A LDLT (2.08%). All thromboses occurred in the 58 incipient patients (63 grafts) with an incidence of 6.34%. Thereafter, HAT did not occur in 124 consecutive cases. HAT was suspected in 14 patients without arterial inflow by routine color Doppler ultrasonographic examination and confirmed in 4 patients by CEUS and HA angiography. These results are shown in Table 4. HAT occurred in 2 recipients on the 1st and 7th d following A-A LDLT, both of which were revascularized with GSV between the donor RHA and recipient AA immediately after thrombectomy. These patients were discharged with good liver function. The third HAT occurred on the 3rd d after A-A LDLT, and urgent administration of thrombolytic therapy combined with anticoagulant treatment was performed and coagulation status was monitored. Despite recovering HA inflow detected by color Doppler ultrasonography with good liver function, the patient died on postoperative day 36 with multiorgan failure (MOF). The fourth HAT occurred on the 46th postoperative day, however, no ischemia-related complication was observed during the follow-up period. No special treatment for this patient was performed because liver function test results and general condition were good.

DISCUSSION

Hepatic arterial reconstruction, which is critical to successful outcome, is one of the most difficult procedures in A-A LDLT. Currently, many scholars emphasize the importance of microvascular techniques for hepatic arterial reconstruction in LDLT to overcome the risk of HAT[14-17]. When comparing microsurgical techniques with conventional methods, the use of microsurgical techniques in HA reconstruction can result in a lower incidence of HAT compared with conventional procedures[17].

Despite the improvements in surgical techniques, a liver graft with a fine HA less than 2 mm in diameter is regarded as a contraindication for LDLT because of the high risk of HAT[15]. As shown in Table 3, the mean diameter of graft arteries and recipient arteries were equal to or less than 2 mm. In our study, all anastomoses were carried out by a group of vascular surgeons, including two experts in vascular surgery and two assistants, using atraumatic microvascular techniques during the reconstructions. Only 4 cases of HAT in recipients occurred which were in the 58 incipient patients following A-A LDLT. As we accumulated experience in microsurgical hepatic arterial reconstructions, no further cases of HAT occurred in 124 consecutive cases following A-A LDLT. On the basis of our experience in LDLT, the key points in HA reconstruction included: (1) selecting a reciprocal stump location for arterial reconstruction: a thicker arterial stump in the recipient was chosen for the first anastomosis. In 58 incipient cases, the primary candidates for the first anastomosis were the RHA and LHA, which are often small in diameter. To increase the blood flow through the anastomosis, a thicker arterial stump in the recipient, close to the donor stump, was used as the first option for anastomosis in the subsequent 124 cases. In order to expand the anastomotic stoma, the caliber discrepancy between the recipient and graft HA was resolved by cutting the smaller arterial end obliquely to change the effective caliber which was twice as wide as the diameter of the smaller vessel. Thereafter, the incidence of HAT decreased markedly. Other methods such as the fish-mouth method, funnelization method, or end-to-side anastomosis were not used in this study; (2) utilizing back wall-first anastomosis technique. Run-

Table 4 Management and outcome of HAT after A-A LDLT

Case	Gender	Age (yr)	Graft	Diagnosis and management time post A-A LDLT (d)	Management	Survival
1	Male	43	RL	1, 1	Revascularized	Alive
2[1]	Male	57	RL + LLS	7, 7	Revascularized	Alive
3	Male	40	RL	3, 3	Medications	Dead
4	Female	33	RL	46	Observation	Alive

[1]HAT occurred in right hepatic artery. HAT: Hepatic arterial thrombosis; A-A LDLT: Adult-to-adult living donor liver transplantation.

ning microsutures were placed in the back wall of the artery first without turning over the vessel. Compared with conventional sutures, running sutures can avoid repeatedly tied and twisting vessels, both of which can injure fragile intima. Running sutures were employed in all HA reconstruction procedures with a low incidence of HAT (2.08%) in our study. Controversially, another report suggested that interrupted sutures may be a better choice for HA anastomosis with a lower incidence of HAT than continuous sutures[26]; and (3) treating intimal dissection of recipient HA. In our study, direct end-to-end anastomosis between the recipient and graft HA was employed in most procedures of A-A LDLT. In the case of intimal dissection, recipient HA is not suitable for hepatic arterial reconstruction. Several methods of revascularization have been reported, including application of recipient splenic or right gastroepiploic artery[27-29], interposition of a vessel graft between the CHA and graft artery[30,31], or between the AA and the graft artery[32]. Despite several reports[4,33] suggesting that interposition of a vessel graft increased the risk of HAT, interposition of GSV was performed between the graft RHA and recipient CHA in 3 cases, and bypass was performed between the graft RHA and recipient AA using GSV in 2 patients and cryopreserved cadaveric iliac vessels in 2 cases. No HAT occurred in the interposition cases. These cases provided a safe and effective approach for the management of intimal dissection. On account of a relatively small sample size in this study, further research is needed.

HAT is the most common vascular complication. Due to irreversible ischemic damage of hepatocytes and bile duct epithelial cells, HAT can cause devastating complications with a high mortality of 50%, including bile leakage, hepatic necrosis, graft loss, and sepsis[4,5]. Several factors were suggested to cause HAT after LDLT: technical problems[34-36], anatomic variation[37], hypercoagulable state[38], severe intraoperative hypotension[38], long cold ischemic time[39], occlusion of HA outflow owing to hepatic congestion[10], and systemic infection[10]. Diagnosis can be suggested by Duplex ultrasonography[12], and confirmed by angiography[40] or CEUS[13]. In our study, among the 14 cases without arterial inflow at routine color Doppler ultrasonographic examinations, CEUS showed 4 cases of HAT that were confirmed by HA angiography which is the gold standard for diagnosing HAT.

In the case of early HAT, several methods[8,9,11], including retransplantation, urgent revascularization, or revision of anastomosis, have been performed to avoid irreversible damage of the allograft. In our study, two recipients underwent urgent revascularization immediately after HAT was confirmed on the 1st and 7th d after A-A LDLT, and were subsequently cured with satisfactory liver function. One HAT patient received thrombolytic and anticoagulant therapy with monitoring of coagulation status, and thereafter HA inflow returned to normal, however, this patient died of MOF on the 36th d after A-A LDLT. Thrombolytic therapy and anticoagulant therapy for HAT should be carried out cautiously due to the potential risk of hemorrhage. Late HAT, which occurs after the first postoperative month, can be well tolerated, and portal flow can support the liver until the collateral circulation has developed[41]. No special treatment for the patient in our study with late HAT was performed because liver function test results and general condition were good. However, long-term results of late HAT patients will require further research.

In conclusion, selecting an appropriate anastomotic artery and applying microvascular techniques for HA reconstruction are crucial to reduce the high risk of HAT during A-A LDLT. The principles of HA reconstruction will be followed in our future clinical procedures which include: a settled group of vascular surgeons, running sutures with intimal eversion, sufficient large caliber of the stoma and atraumatic manipulation. Running sutures may be a reliable alternative for anastomosis of small diameter HAs with a low incidence of HAT following A-A LDLT. Nevertheless, randomized prospective controlled studies should be performed to validate these results.

COMMENTS

Background

Hepatic artery thrombosis (HAT), which is the most common vascular complication after living donor liver transplantation (LDLT), can result in graft loss and devastating consequences. Surgical techniques are suggested to an important factor in causing HAT. Despite improvements in surgical techniques, arterial reconstruction in LDLT has a high risk of thrombosis.

Research frontiers

Although microsurgical techniques in arterial reconstruction has helped overcome the high risk of HAT, because of the short donor hepatic artery (HA) stump, which is small in diameter, and vessel size discrepancy between the graft and recipient hepatic arteries, reconstruction of hepatic arteries is a challenge to surgeons in LDLT. The incidence of HAT after LDLT varies widely from 4%-25%.

Innovations and breakthroughs

With atraumatic microsurgical techniques, the reconstruction of HA was accomplished in 182 recipients (192 grafts) of adult-to-adult LDLT performed

from January 2001 to September 2009 by a settled group of vascular surgeons. Interposition of the great saphenous vein or cryopreserved vessel between the recipient and graft was performed for the treatment of 7 intimal dissections of recipient hepatic arteries. Both back-wall first techniques and running sutures with intimal eversion were employed in all anastomoses, and a good outcome with a low incidence of HAT (2.08%), was achieved.

Applications

Besides the use of microsurgical techniques, reconstruction of the HA by settled surgeons in LDLT provides an approach for decreasing the incidence of HAT. Based on our study results, running sutures can be used in the anastomoses of hepatic arteries of small diameter.

Peer review

This is a retrospective analysis of HA thrombosis after living donor liver transplantation.

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CASE REPORT

Perineal rectosigmoidectomy for gangrenous rectal prolapse

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Abstract

Incarceration rarely complicates the chronically progressive form of the full thickness rectal prolapse. Even more rarely, it becomes strangulated, necessitating emergency surgery. We describe an extremely rare case of incarcerated acute rectal prolapse, without a relevant previous history or symptoms of predisposing pathology. The patient underwent emergency perineal proctosigmoidectomy, the Altemeier operation, combined with diverting loop sigmoid colostomy. The postoperative course was quite uneventful with an excellent final result after colostomy closure. The successful treatment of this patient illustrates the value of the Altemeier procedure in the difficult and unusual case scenario of bowel incarceration.

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Key words: Anorectal disease; Rectal prolapse; Incarceration; Perineal rectosigmoidectomy; Altemeier operation

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INTRODUCTION

Rectal prolapse (RP) is a rare anorectal disease with underestimated incidence, affecting both children and adults. Although known and described as early as 1500 BC[1], there is still uncertainty concerning its clinical definition, course and pathophysiology which justifies the numerous therapeutic modalities and operations proposed.

Three different clinical entities may coexist or be combined and inclusively called RP: the mucosal prolapse (partial or pseudoprolapse), the internal prolapse (rectal intussusception) and the full thickness RP (complete or true)[2]. The mucosal prolapse concerns the protrusion of the rectal mucosa usually as a result of a lesion such as hemorrhoids or polyps. Internal intussusception may be a full thickness or a partial wall disorder but the prolapsed tissue does not pass beyond the anal canal. Lastly, full thickness RP is a complete protrusion of the rectum (and/or the sigmoid) with its entire wall through the anus. Its course is usually progressive; initially it is reducible spontaneously, later manually and is finally irreducible. In any stage it may be complicated by incarceration and strangulation.

We describe a case of a young male patient presenting with acute, incarcerated large RP who was successfully managed by perineal proctosigmoidectomy (Altemeier procedure) combined with diverting sigmoid loop colostomy.

CASE REPORT

A previously fit, healthy and normal weight (body mass index, 27.8) 37-year-old Albanian male presented to our emergency department with a large, incarcerated RP resulting from intense straining 10 h before admission (Figure 1). Another episode of RP with easy manual reduction at the age of 10 years was vaguely mentioned in the patient's history. Neither chronic constipation, nor a relevant family history was reported. On clinical examination, the patient had a normal temperature and was hemodynamically stable and normal. Abdominal examination revealed mild tenderness in the lower abdomen while the laboratory investigation showed mild leucocytosis with left shift. As the bowel was not obviously necrotic, conservative measures were applied in order to permit repositioning of the bowel. Unfortunately, sedation, placing of the patient in the Trendelenburg position and topical application of warm compresses and 20% mannitol, all were unsuccessful and the patient was taken to the operating room after 4 h of ineffective efforts.

Under general anesthesia, a dilute epinephrine solution was injected 2-3 cm proximal to the dentate line and the rectal wall was incised across its full thickness at this level. The vessels of the mesorectum and mesosigmoid were carefully ligated, the prolapsed rectosigmoid was resected and a hand-sewn interrupted coloanal anastomosis in 2 layers was created. In addition, a loop sigmoid colostomy for a protective and fixing purpose was constructed. The patient's postoperative course was quite uneventful and the patient was discharged on the 10th postoperative day. Histological stained sections revealed severe ischemic alterations. The vessels of the bowel wall were found either dilated or thrombosed while the mucosa had multiple regions of necrosis and ulceration.

Six months later, the patient was readmitted for colostomy closure after physiologic examination with anal manometry, which was normal. However, a preoperative colonoscopy showed intense inflammatory changes at the anastomotic suture line, due to incompletely absorbed suture material plus a remnant rectal colitis. Short term conservative treatment with enemas and suppositories of mesalamine was successfully applied for a 20-d period. Both revision colonoscopy and barium enema, performed 1 mo later, were normal and the colostomy was closed without further complications. Ever since, and for an 8 mo period, the patient has been well.

DISCUSSION

There is still some debate about the exact pathophysiologic mechanism of RP. The prevailing theories are those of sliding herniation and progressive internal intussusception. Chronic or, less frequently, acute abdominal straining is present in most cases[3]. In addition, both pathologies coexist with different anatomical and physiological features (patulous anus, incontinence) and common predisposing factors (straining, constipation)[4]. The most usual form of RP is the chronic course of the disorder which permits selective use of investigative tools like sigmoidoscopy, videoproctography or, more rarely, physiologic studies such as anal manometry. It is evident that all diagnostic efforts are practically omitted in the rare scenario of incarcerated or strangulated RP, where urgent surgical treatment becomes a priority[5].



Figure 1 Patient with a large, incarcerated rectal prolapse in the operating room.

A wide spectrum of operative procedures are available mainly for elective cases[6]. They are categorized as resective, fixative or a combination of both in order to achieve 2 goals: anatomical repositioning of the bowel and improvement of the function of the anorectal complex. The approach may be either abdominal or perineal. The selection of the appropriate procedure primarily depends on the patient's clinical data adjusted to the operator's experience and personal preference[7].

Abdominal approaches are performed in patients fit enough to tolerate laparotomy as these seem to result in lower recurrence rates[8], perhaps with the exception of young men who cannot afford the increased risk of impotence and infertility from an abdominal operation[9]. In elective cases, rectopexy, using fixing material (mesh, sutures, clips), is the most popular operation with good results concerning recurrence[10,11]. The fixation to the presacral fascia can be done either anteriorly (Ripstein) or posteriorly. However, when constipation dominates symptomatology, usually with coexistence of a redundant sigmoid, an anterior resection alone or in combination with rectopexy is usually preferred[12]. In the modern era of surgery, the above operations can be accomplished laparoscopically with minimal morbidity and mortality[13].

When the prolapse cannot be manually reduced, a few techniques may help the bowel return to its anatomic position. Sedation, Trendelenburg position of the patient and topical application of salt and sucrose may decrease bowel edema and enable reduction[9,14]. Even the use of an elastic compression wrap has been practiced[15]. However, when the prolapsed bowel is incarcerated or strangulated and cannot return to its anatomic position by the measures described above, the situation becomes a surgical emergency, as was the case with our patient. Jeopardizing the bowel's viability should be avoided as gangrene significantly increases morbidity and mortality and thus, an urgent surgical intervention is

always indicated[16,17]. The operation of choice is perineal proctosigmoidectomy with or without colostomy[16]. Ramanujam *et al*[17] described 8 cases of acute incarcerated RP during a 9-year period, where perineal rectosigmoidectomy was performed. All were women over the age of 75 and 2 (25%) developed postoperative anastomotic leak with pelvic peritonitis requiring diverting colostomy.

Our male patient, with acute presentation of incarcerated RP, underwent emergency perineal resection as the only alternative to remove the ischemic bowel. Goligher states that irreducibility with gangrene remains one of the few indications for rectosigmoidectomy (perineal) at the present time[18]. Furthermore, in recent years, there has been a trend towards offering elective perineal rectosigmoidectomy in healthier younger patients especially in males[8]. The abdominal approaches carry an increased risk of impotence and infertility when comparing to the perineal ones[9]. Unfortunately, the recurrence rate after the Altemeier operation is not negligible. There have been historical reports of a recurrence rate of up to 58%[19]. More recent studies refer to much lower rates (3%-16%), which are still high when compared to the abdominal approaches[8,20]. In addition, restoration of continence following the operation is also unpredictable as it may result in increased soiling and frequency of defecation[8]. Therefore, the addition of levatorplasty to perineal rectosigmoidectomy has been suggested in order to achieve better results[8].

A transabdominal construction of a sigmoid loop colostomy was added aiming to protect the "difficult" hand sewn anastomosis from the fecal stream. The excessive bowel edema due to incarceration made the operator consider the anastomosis unsafe. In addition, colostomy might also have a fixing function, but, on the other hand, carries various risks and complications[21]. The inflammatory changes and remnant colitis seen in this patient's colonoscopy were rather minor and they were successfully dealt with by pharmacologic means.

In conclusion, a rare case of incarcerated and strangulated RP in a young adult is described in our case report. The patient's successful treatment with perineal proctosigmoidectomy highlights the value of the Altemeier procedure in this emergency situation.

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CASE REPORT

Solitary concomitant endocrine tumor and ductal adenocarcinoma of pancreas

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Abstract

Pancreatic tumors with combined exocrine and endocrine features are rare. Most reported cases are classified as mixed exocrine and endocrine carcinoma of the pancreas. We report the first case of solitary concomitant endocrine tumor and ductal adenocarcinoma of the pancreas. A 58-year-old patient was admitted for uncontrolled diabetes mellitus and body weight loss. The tumor was fortuitously discovered in the pancreatic tail after a tumor survey panel. Grossly, the solitary tumor had a central fibrous band that clearly divided it into two parts. On microscopic examination, the tumor contained both endocrine and exocrine components distinctly separated by the central fibrous band. The exocrine part showed a poorly-differentiated adenocarcinoma. The endocrine part was strongly immunoreactive to chromogranin, synaptophysin and glucagon. We reviewed the literature on pancreatic tumors with combined exocrine and endocrine features. A simple classification for this group of neoplasms is suggested, including five types: amphicrine, mixed, collision, solitary concomitant and multiple concomitant.

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Key words: Pancreas; Concomitant tumor; Collision tumor; Mixed tumor; Glucagonoma

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INTRODUCTION

Pancreatic tumors, categorized according to exocrine and endocrine origin, are classified into ductal, acinar or endocrine types by morphological and immunohistochemical studies. Pancreatic endocrine tumor (PET) is uncommon and represents only 1%-2% of all pancreatic tumors[1]. Pancreatic exocrine tumor is rarely associated with pancreatic endocrine tumor[2]. Most reported cases are mixed ductal-endocrine carcinoma, mixed acinar-endocrine carcinoma and mixed ductal-acinar-endocrine carcinoma[2-7]. These reported cases are classified accord-

ing to World Health Organization (WHO) criteria[8], in which mixed exocrine and endocrine carcinoma is defined as carcinoma containing intimately intermixed exocrine and endocrine components, and each component comprises at least one-third of the tumor tissue. By this definition, a solitary concomitant pancreatic tumor with distinctly separated exocrine and endocrine components cannot be classified as mixed exocrine and endocrine carcinoma. Here, we report a case of solitary concomitant endocrine tumor and ductal adenocarcinoma of the pancreas. This type of pancreatic tumor is especially rare and, to the best of our knowledge, has never been reported in the English literature.

CASE REPORT

A 58-year-old Chinese male was admitted to the endocrinology and metabolism ward in July 2009 for poorly controlled hyperglycemia, which was refractory to an oral hypoglycemic agent. He had a history of diabetes mellitus (DM), hypertension and chronic renal insufficiency for more than 4 years and had been receiving regular follow-up at the outpatient clinic of this hospital. On admission, laboratory test (Table 1) revealed that his blood glucose was 561 mg/dL. Physical examination showed no skin rash or oral lesions. While he was hospitalized, daily high-dose insulin injection with Novomix 60 U/d was necessary to control his blood glucose level. The patient also complained of losing 10 kg of body weight in the prior two months. Therefore, he asked for a tumor marker examination. A tumor survey panel revealed an elevated carbohydrate antigen 19-9 (CA19-9) of 195 U/mL (normal, 0-37 U/mL) incidentally. Abdominal computed tomography (CT) demonstrated a 3.7 cm × 2.1 cm tumor in the pancreatic tail (Figure 1) and unremarkable adrenal glands bilaterally. Whole body positron emission tomography showed moderately increased accumulation of fluorine-18 fluorodeoxyglucose (F-18 FDG) in the pancreatic tail with standard uptake values of 3.3 (initial) and 4.5 (delayed). No increased uptake in other organs or sites was identified. Thyroid ultrasound showed no focal lesion. The patient received distal pancreatectomy and splenectomy under the impression of pancreatic tail carcinoma. Neither liver metastasis nor peritoneal seeding was found during surgery. The postoperative course was uneventful. Initially, only an oral hypoglycemic agent was prescribed for controlling DM when the patient was discharged from the hospital. However, because hyperglycemia (268 mg/dL) and elevated glycosylated hemoglobulin HbA1c (8.1%) were found at follow-up in the outpatient clinic, insulin injection with Novomix 28 U/d was represcribed. The patient moved to another city for family support and admitted there one month after operation. Therefore we lost the follow-up of him.

Grossly, the surgical specimen consisted of pancreatic tail tissue, 10 cm × 4 cm × 3.5 cm, and unremarkable spleen tissue (146 gm). A well-circumscribed gray-brown nodular mass, 3.8 cm × 3.2 cm × 2.2 cm, was found in



Figure 1 Computed tomography (CT) scan demonstrating a 3.7 cm × 2.1 cm tumor (arrow) in pancreatic tail.

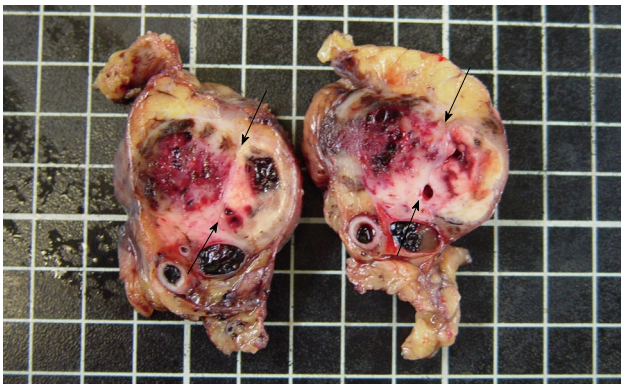


Figure 2 Gross examination showing a well-circumscribed tumor with a central fibrous band (arrows) separating the right exocrine and the left endocrine parts.

the pancreatic parenchyma. On serial cuts, the nodular mass clearly showed a red-brown, hemorrhagic, centrally necrotic heterogenous part and a gray-white, focally hemorrhagic solid part (Figure 2). A thin fibrous band was present between these two parts. No gross invasion to spleen, large vessels or resection margins was found.

The microscopic sections showed pancreatic tissue with a partially encapsulated tumor composed of two distinctly separated, exocrine and endocrine components (Figure 3A). The two components did not grow into each other and were completely separated by a small band of fibrous tissue and some residual, atrophic, benign exocrine glands. The endocrine part, up to 1.8 cm in diameter and occupying more than 40% of the pancreatic tumor, was well-circumscribed, completely enclosed by fibrous tissue and composed of uniform tumor cells containing small, round, monotonous nuclei and pale or mildly acidophilic cytoplasm (Figure 3B). These cells were arranged in gyriform or trabecullar patterns divided by slender fibrous connective tissue and rich vascular stroma. Neither mitotic figures nor cellular atypia was found. No evidence of necrosis, extrapancreatic spread or vascular invasion was present. Mucin and congo red stains were negative. On immunohistochemical (IHC) staining, the tumor cells were diffusely and strongly

Table 1 Laboratory data on admission

CBC and DC		Biochemistry data		Tumor marker	
WBC	13460/mm^3	Glucose	561 mg/dL	CA19-9	195 U/mL
RBC	5.11 × 10^6/mm^3	Albumin	2.7 g/dL	AFP	6.38 ng/mL
Hemoglobin	15.2 g/dL	Sodium	136 mmol/dL	CEA	2.01 ng/mL
Hematocrit	44.9%	Potassium	3.25 mmol/dL	PSA	1.90 ng/mL
Neutrophil	79.0%	BUN	48 mg/dL		
Lymphocyte	16.1%	Creatinine	1.6 mg/dL		
Monocyte	3.9%	Calcium	2.08 mmol/L		
Eosinophil	0.7%	Triglyceride	133 mg/dL		
Basophil	0.3%	Cholesterol	85 mg/dL		
Platelet	32.7 × 10^4/mm^3				

Normal values: CA19-9: 0-37 U/mL; AFP: 0-10 ng/mL; CEA: 0-7 ng/mL; PSA: 0-4 ng/mL. CBC and DC: Complete blood cell count and differential count; WBC: White blood cell count; RBC: Red blood cell count; BUN: Blood urea nitrogen; CA19-9: Carbohydrate antigen 19-9; AFP: α-fetoprotein; CEA: Carcinoembryonic antigen; PSA: Prostate-specific antigen.

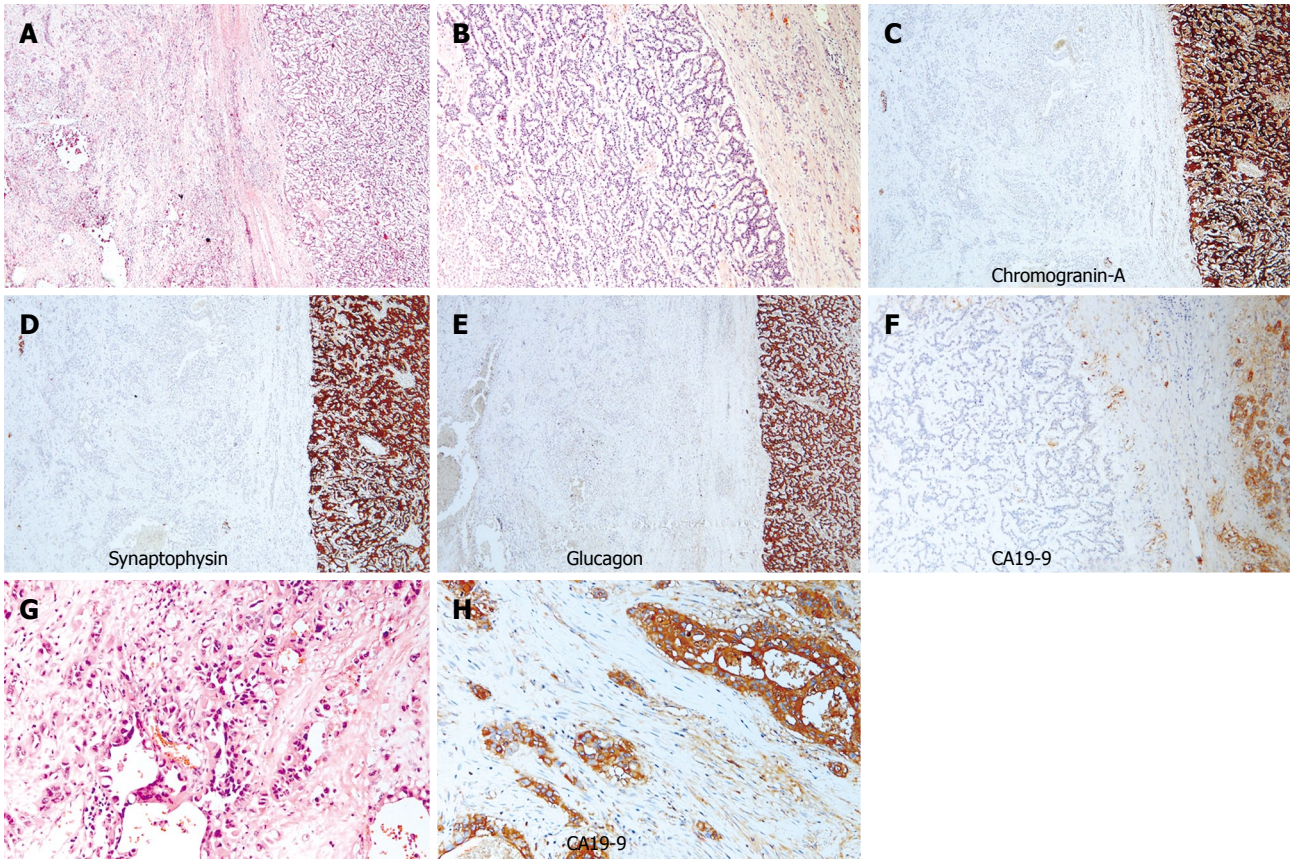


Figure 3 Microscopic features of solitary concomitant endocrine tumor and ductal adenocarcinoma of pancreas. A: Low-power view showing ductal adenocarcinoma (left) and endocrine tumor (right) with a fibrous band (central) clearly demarcating the two components (HE × 40); B: Pancreatic endocrine tumor composed of monotonous small, round tumor cells arranged in gyriform or trabecular patterns, marginated by a fibrous wall (right) (HE × 100); C-E: Pancreatic endocrine tumor (right) showing positive expression while ductal adenocarcinoma (left) showing negative expression of chromogranin A, synaptophysin and glucagon (IHC × 40); F: Pancreatic endocrine tumor (left) showing negative expression while residual benign pancreatic glands (right) showing positive expression of carbohydrate antigen 19-9 (CA19-9) (IHC × 100); G and H: Higher magnifications of ductal adenocarcinoma showing bizarre pleomorphic glands (HE × 200) and immunoreactive to CA19-9 (IHC × 200).

positive for chromogranin A (Figure 3C), synaptophysin (Figure 3D) and glucagon (Figure 3E). No expression of CA19-9 (Figure 3F), p53, Bcl-2, insulin, gastrin, vasointestinal peptide was demonstrated in the tumor. Ki-67 + cells were less than 0.5%. The exocrine component, a poorly-differentiated ductal adenocarcinoma, showed bizarre and hyperchromatic cells containing pleomorphic darkly staining nuclei, some mitoses and pale or acidophilic cytoplasm (Figure 3G). Scattered anaplastic nuclei were also found. These tumor cells were arranged in dilated ductal structures, small glands, cellular files or single cells, with large areas of necrosis and hemorrhage.

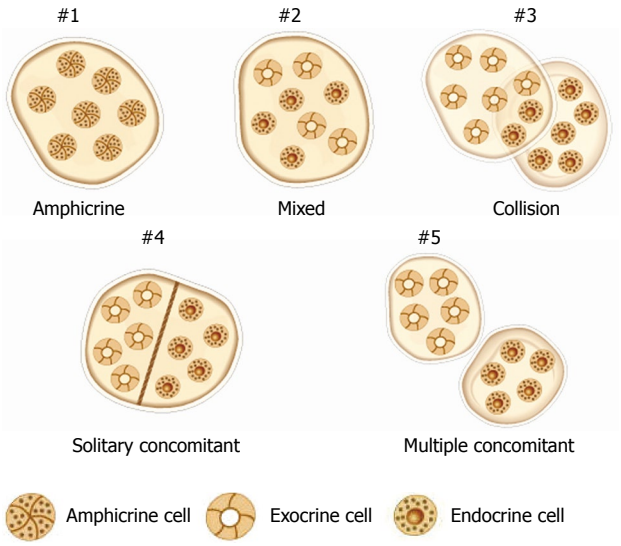


Figure 4 Five types of pancreatic neoplasm with combined exocrine and endocrine features.

The exocrine component showed focal invasion of peripancreatic soft tissue. The spleen and 9 peripancreatic lymph nodes were free of tumor. Mucin secretion was demonstrated by mucicarmine and periodic acid-Schiff with diastase stains. On immunohistochemical staining, this component was strongly positive for CA19-9 (Figure 3H), and negative for chromogranin A (Figure 3C), synaptophysin (Figure 3D), glucagon (Figure 3E), insulin, gastrin, vasointestinal peptide and Bcl-2. Ki-67 + cells were more than 25%. More than 55% of the ductal adenocarcinoma had strong intranuclear accumulation of p53 protein. In this case, the exocrine and endocrine components were clearly separated and different from each other in the single tumor. No collision or intermixing area could be found. With no other endocrine manifestations and focal lesion found in the thyroid and adrenal glands, MEN type 1 was not considered. Finally, a solitary concomitant endocrine tumor and ductal adenocarcinoma of the pancreas was diagnosed.

DISCUSSION

To the best of our knowledge, this is the first case of solitary concomitant PET and ductal adenocarcinoma of the pancreas reported in the English literature. Ballas *et al*[9] reported a case of mixed exocrine-endocrine tumor of the pancreas showing two distinct populations of tumor cells. Most parts of these two components were separated but focally showed a collision type of growth, with two different components growing into one another. Goh and colleagues[10] reported three cases of concomitant intraductal papillary mucinous neoplasm (IPMN) of the pancreas and pancreatic endocrine neoplasm. The sizes of the endocrine part were small (2, 5 and 8 mm) and well-circumscribed, isolated and nonfunctional. However, the morphology of all three exocrine components was not indicative of classical invasive ductal carcinoma as the present one.

Many reports have discussed the histogenesis of the association between exocrine and endocrine neoplasms of the pancreas[2,3,11,12]. Leteurtre *et al*[3] reported a case of mixed ductal-endocrine carcinoma of the pancreas and supported the hypothesis of a common endodermal histogenesis for ductal and endocrine cells. Chatelain and colleagues[2] also suggested that these tumors probably arise from a totipotent endodermal cell distributed in the pancreatic ducts and islets. Marrache *et al*[11] presented 6 patients with associated IPMN and PET, and suggested that these tumors may arise from a common progenitor cell or due to differentiation from one cell type to the other. Morikane *et al*[12] presented a small glucagonoma of the pancreas with evident ductular and tubular structures and supported the hypothesis that pancreatic endocrine tumors originate in the ductular epithelium. In the report of Cho *et al*[13], the tumor cells of a mixed acinar-endocrine carcinoma of the pancreas showed amphicrine differentiation, with one cell type harboring both exocrine and endocrine markers simultaneously, indicating that a close histogenetic relationship exists between pancreatic exocrine and endocrine cells. Pour *et al*[14] concluded that pancreatic cancer derives from pancreatic stem cells distributed within ductal trees and islets. According to the previous literature, it seems reasonable to hypothesize that associated exocrine and endocrine neoplasms of the pancreas arise from totipotent pancreatic stem cells, which reside in the pancreatic duct and islets. Under certain stimulation, the stem cells differentiate and give rise to exocrine and endocrine primitive cells. The exocrine primitive cells further differentiate into ductal and acinar cells. According to the proposed differentiation pathway, we hypothesize that the amphicrine type arises from early stem cells that proliferate into a neoplasm before differentiating into endocrine and exocrine cells (Figure 4). Thus, the amphicrine type, as in the case reported by Cho *et al*[13], contains only one cell type that has both exocrine and endocrine features. When the stem cells begin to differentiate into primitive exocrine and endocrine cells, three types of histological patterns may occur: mixed, collision and solitary concomitant. In the mixed type, the exocrine and endocrine cells are intimately intermixed together. Most reported cases of mixed exocrine and endocrine tumor of the pancreas are examples of this type[2-7]. The collision type, as in the case reported by Ballas *et al*[9], shows the endocrine part at one end and the exocrine part at the other end, with an intermixed central zone. The solitary concomitant type, first presented in our case, shows a single tumor composed of distinctly separate exocrine and endocrine components, with a dividing fibrous band found between the two parts. The last type is a multiple concomitant type that contains isolated exocrine and endocrine tumors simultaneously. The three cases reported by Goh *et al*[10] can be classified as the early ones of this latter type.

Because the present tumor contained both pancre-

atic endocrine tumor and exocrine carcinoma, we tried to consider these two parts together when discussing the prognosis of this patient. No evidence of metastasis was found at abdominal CT, whole body positron emission tomography scan and during surgery. The size of the present endocrine tumor was 1.8 cm. The microscopic features showed scarce mitoses and less than 0.5% Ki-67+ cells. Neither extrapancreatic spread nor vascular invasion was identified. According to criteria of the WHO Committee for Histological Typing of Endocrine Tumors, the present endocrine tumor was classified as a well-differentiated endocrine tumor with benign behavior[1]. In the previous literature and papers presenting cases of mixed exocrine and endocrine carcinoma, the authors suggested that the prognosis depends mainly on the stage of ductal carcinoma[2,8,9]. We cannot be certain that the prognosis of our case depends on the ductal carcinoma component because this is not a case of mixed exocrine and endocrine carcinoma of the pancreas as previously presented. Further study with more cases is necessary.

Chatelain *et al*[2] presented a case of mixed ductal-pancreatic polypeptide-cell carcinoma of the pancreas. In their study, the endocrine cells were not immunoreactive to p53 and Bcl-2, but the exocrine ductal cells were stained strongly positive, suggesting that over-expression of p53 and Bcl-2 plays a major role in neoplastic progression of the ductal component. We performed p53 and Bcl-2 immunostains in the present case. The endocrine part was negative for p53 and more than 55% of exocrine neoplastic cells showed strong intranuclear accumulation. However, both the endocrine and exocrine parts were negative for Bcl-2. The role of p53 and Bcl-2 in tumor histogenesis still needs further investigation.

Glucagonomas are rare and only represent about 10% of all functioning pancreatic tumors[15-17]. Cases without typical symptoms usually remain undiagnosed, resulting in long-term medical waste, and mental and physical suffering of the patients[18,19]. In our case, the patient only presented with DM and body weight loss and did not satisfy the criteria of glucagonoma syndrome. However, it was not certain whether the alpha-cell tumor plays a role in the manifestation of DM or not. In a recent study of pancreatic cancer-associated DM, Pannala *et al*[20] concluded that pancreatic cancer is a powerful diagnostic state and new-onset DM is likely induced by the tumor. Under this viewpoint, the adenocarcinoma of our case may be closely related to the poor control of DM, especially because the patient did not develop glucagonoma syndrome. In short, we suggest that, in cases of type Ⅱ DM occurring in middle-aged patients, the development of uncontrolled hyperglycemia should raise clinical suspicion of glucagonoma and/or pancreatic cancer. In this situation, further survey including pancreatic hormones and tumor marker profile is suggested.

In conclusion, combined pancreatic endocrine and exocrine tumors are rare. We have reported the first case of solitary concomitant pancreatic endocrine tumor and ductal adenocarcinoma. We suggest a classification system of tumor histogenesis for this group of tumors, including amphicrine, mixed, collision, solitary concomitant and multiple concomitant. Further investigation of more cases of this rare entity is still necessary.

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CASE REPORT

Huge pelvi-abdominal malignant inflammatory myofibroblastic tumor with rapid recurrence in a 14-year-old boy

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Abstract

Inflammatory myofibroblastic tumor (IMT) is an uncommon benign neoplasm with locally aggressive behavior but malignant change is rare. We report an unusual case of pelvic-abdominal inflammatory myofibroblastic tumor with malignant transformation in a 14-year-old boy presenting with abdominal pain and 9 kg body weight loss in one month. Computed tomography revealed a huge pelvi-abdominal mass (30 cm), possibly originating from the pelvic extraperitoneal space, protruding into the abdomen leading to upward displacement of the bowel loops, downward displacement of the urinary bladder, massive central necrosis, a well-enhanced peripheral solid component with prominent peritumoral vascularity. Subsequent examination confirmed the computed tomographic findings. Histopathologic examination revealed proliferative epitheloid and spindle cells, inflammatory cell infiltration and high mitotic counts. Immunohistochemistry was strongly positive for anaplastic lymphoma kinase and revealed a high proliferative index (ki-67 = 40%). DNA sequencing and electronic microscopy further confirmed the primitive fibroblastic cell phenotype of the tumor and a final diagnosis of inflammatory myofibroblastic tumor with malignant transformation was established. Rapid tumor recurrence was noted 20 d after radical tumor resection. To our knowledge, this is the largest documented case of IMT in a pediatric patient and the first report of IMT with malignant transformation originating from the pelvic extraperitoneal space.

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Key words: Inflammatory myofibroblastic tumor; Malignant transformation; Pediatric patient; Pelvis; Extraperitoneal space; Computed tomography

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Lu CH, Huang HY, Chen HK, Chuang JH, Ng SH, Ko SF. Huge pelvi-abdominal malignant inflammatory myofibroblastic tumor with rapid recurrence in a 14-year-old boy. *World J Gastroenterol* 2010; 16(21): 2698-2701 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i21/2698.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i21.2698

INTRODUCTION

Inflammatory myofibroblastic tumor (IMT) is an uncommon benign neoplasm with locally aggressive behavior and a tendency to recur[1-10]. An IMT is commonly found

in the lung as an asymptomatic solitary lung mass detected on chest radiography[1-3]. Extrapulmonary IMT can occur in nearly every site in the body but is most commonly found in the mesentery and omentum, orbit, and gastrointestinal and genitourinary tracts[1-4]. IMT of the retroperitoneum is unusual[3-6]. On rare occasions, IMT may exhibit malignant transformation or may evolve into lymphoreticular malignancy[2,3,7]. Herein, we describe an extremely unusual case of IMT with malignant transformation originating from the pelvic extraperitoneal space in a pediatric patient.

CASE REPORT

A previously healthy 14-year-old boy presented with abdominal pain and fullness with body weight loss of 9 kg in one month. A physical examination revealed slightly pale conjunctiva, a 23 cm × 33 cm fixed pelvi-abdominal mass and engorged superficial veins on the abdominal wall. Laboratory investigations showed that hemoglobin was 11.8 g/dL and the leukocyte count was 13.3 × 10^9/L with eosinophilia (eosinophil count 17.8%), and an elevated C-reactive protein level (77.7 mg/L). Computed tomography (CT) showed a huge pelvi-abdominal mass (30 cm), possibly originating from the pelvic extraperitoneal space, protruding into the abdomen with upward displacement of the bowel loops, downward displacement of the urinary bladder, massive central necrosis, and a well-enhanced peripheral solid component with prominent peritumoral vascularity (Figure 1A-C). Based on such CT features, the surgeons were notified for cautious surgical dissection of the extraperitoneal space as well as the possibility of massive bleeding and the need for blood transfusions during the operation. The initial impression included mesenteric fibromatosis, IMT, gastro-intestinal stromal tumor or mesenteric sarcoma. During surgery, CT findings were confirmed and prominent peritumoral vascularity was encountered resulting in a blood loss of 3900 mL during radical tumor resection and necessitated transfusion of packed red blood cells (6 units) and fresh-frozen plasma (4 units). The gross specimen was a slightly lobulated mass with an uneven peripheral solid component and massive central necrosis (Figure 1D). Histopathologic examination revealed predominantly epitheloid and spindle cells, prominent staghorn vascular channels, inflammatory cell infiltration and high mitotic counts (14/10-high-power-field) (Figure 1E). Immunohistochemistry was strongly positive for anaplastic lymphoma kinase (ALK) and revealed a high proliferative index (ki-67 = 40%) but was negative for desmin, HHF-35, Fli-1, CD31, CD34, CD117, S-100, chromogranin A, synaptophysin, and P-53. Real-time polymerization chain reaction assay revealed strong expression of TPM3-ALK fusion transcript and DNA sequencing revealed ALK fusion gene breakpoint at TPM3 Exon 8 to ALK Exon 21. Electron microscopy of the spindle cells revealed lack of subplasmalemal thin actin filament and microtendon in fibronexa on the cell surface confirming primitive fibroblastic cell phenotype. A final diagnosis of IMT with malignant transformation was established. The patient recovered uneventfully and was discharged 10 d after surgery. Unfortunately, a recurrent palpable mass was noted over the lower abdomen 20 d after discharge. He refused further treatment and died 6 mo later.

DISCUSSION

IMT has been reported as a quasineoplastic lesion histopathologically characterized by proliferation of myofibroblastic spindle cells and inflammatory cells. Most cases of IMT are found in the lung, orbit, mesentery and omentum, and gastrointestinal and genitourinary tracts[1-4]. IMT arising from uncommon locations including the liver, spleen, head and neck, central nervous system, heart, appendix and superficial soft tissue has also been documented[2-8]. Excluding urinary tract lesions, retroperitoneal IMT is unusual and only 10 cases have been described[3-6]. To our knowledge, this is the first reported case of IMT in the pelvic extraperitoneal perivesical space.

The etiology of IMT is contentious and whether it is a post-inflammatory process or a true neoplasm remains controversial[2-8]. Proponents for post-inflammatory or immunologic processes advocate the association of IMT and infectious agents such as mycobacteria, corynebacteria, the Epstein-Barr virus and human herpes virus, as well prior histories of surgery, trauma, and steroid usage[2-5]. In contrast, proponents for neoplasm emphasize the aggressive behavior, cytogenetic clonality, involvement of specific chromosome region 2p23 and the possibility of distant metastasis[5-8]. In this particular case, there was no definite evidence of related infection, a traumatic episode or prior steroid usage. On the other hand, histopathologic and immunohistochemical features such as high mitotic counts, positive ALK and a high proliferative index as well as the rapid recurrence after initial radical resection were supportive of the neoplastic nature of IMT.

The sizes of reported IMT ranged from 0.4 cm to 40 cm[1-10]. An intraabominal IMT is usually larger and the largest reported IMT (40 cm) occurred in the liver of a 29 year-old man[9]. Our patient had the largest reported IMT among pediatric patients. The clinical presentation varies depending on the anatomical location of the lesion[1-10]. As demonstrated in our patient, patients with abdominal IMT usually present with abdominal pain, palpable mass, and body weight loss. Occasionally, features of inflammation such as fever, an elevated erythrocyte sedimentation rate and a markedly elevated leukocyte count (> 80 × 10^9/L) are encountered[10].

The CT appearance of abdominal IMT is variable. The mass may be iso- or hypodense to muscle on unenhanced scans with variable enhancement patterns including homogeneous, heterogeneous, peripheral and delayed central filling patterns[1,4-10]. As seen in our patient, central necrosis may occur in a large abdominal IMT and the differential diagnosis of a large abdominal mass with central necrosis encompasses giant neurilemmoma, gastrointestinal stromal tumor and fibromatosis[11-13]. A giant abdominal neurogenic tumor in a patient without

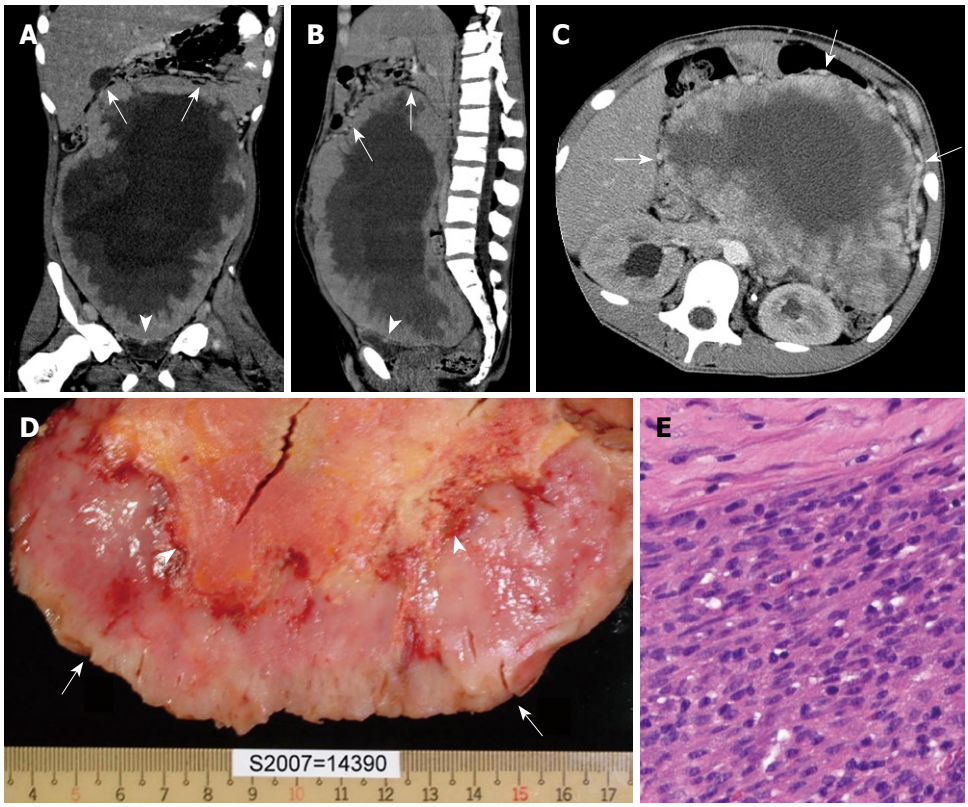


Figure 1 Malignant inflammatory myofibroblastic tumor in a 14-year-old with a history of abdominal pain and body weight loss. A, B: Coronal (A) and sagittal (B) computed tomography (CT) images reveal a huge pelvi-abdominal mass with an uneven peripheral solid component and massive central necrosis, with upward displacement of the bowel loops (arrows) and downward compression on the urinary bladder (arrowheads), highly suggestive of an extraperitoneal tumor originating from the perivesical space; C: Axial CT image at the level of the kidney clearly demonstrates prominent vessels around the tumor (arrows); D: The cut-surface of the gross specimen reveals an uneven peripheral solid component (arrows) and massive central necrosis (arrowheads); E: Photomicrograph shows proliferation of spindle tumor cells in the peripheral solid part (HE stain, original magnification × 200).

von Recklinghausen's disease was unusual and the lack of direct involvement of the gastrointestinal tract rendered gastrointestinal stromal tumor less likely[11,12]. CT demonstration of prominent peritumoral vascularity was suggestive of prominent regional inflammatory changes, supporting the diagnosis of IMT rather than fibromatosis[13]. In this particular case, coronal and sagittal reconstructed CT images clearly depicted the mass effect with upward displacement of the bowel loops and downward displacement of the urinary bladder, implying that the tumor might be extraperitoneally located. This information was helpful for preoperative planning, advocating cautious dissection of the extraperioneal spaces during surgery. In addition, surgeons were forewarned of the possibility of massive bleeding during tumor resection and the necessity for blood transfusion. Although CT may offer suggestive features of this unusual tumor, the definite diagnosis relies on detailed histopathologic and immunohistochemical evaluations[2-10]. ALK is an oncoprotein which essentially serves as a surrogate marker of chromosome rearrangement of 2p23 in IMT. Real-time polymerization chain reaction assay revealed strong expression of TPM3-ALK fusion transcript in our patient and in addition, DNA sequencing revealed ALK fusion gene breakpoint at TPM3 Exon 8 to ALK Exon 21 allowing further confirmation of IMT[14]. Chemotherapy, radiotherapy, and even immunomodulation have not been reported consistently effective against this aggressive tumor[7,8]. Radical resection remains the treatment of choice for patients with IMT and is adequate in most circumscribed tumors. However, mesenteric or retroperitoneal origin of the tumor, large tumor (> 8 cm), multinodular or ill-defined tumor morphology and incomplete tumor resection of IMT are factors associated with higher recurrence rates (17%-37%)[2-6,10]. In this particular patient, despite radical tumor resection being accomplished, rapid tumor recurrence was noted in 20 d and fatality in 6 mo, further confirming the highly malignant nature of the mass. In retrospect, the presence of abundant peritumoral vascularity demonstrated on CT was suggestive of high metabolic demand of the tumor which might be a clue to potential malignant change.

In summary, this report documents the first known case of pelvic extraperitoneal IMT with malignant transformation in a pediatric patient. In light of this case, IMT should be considered in the differential diagnosis of pelvi-abdominal mass with large central necrosis and the presence of prominent peritumoral vascularity may also be a clue of high metabolic demand and even malignant transformation.

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CASE REPORT

Acute cholecystitis immediately after radical gastrectomy: A report of three cases

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Abstract

Acute cholecystitis is not a common complication of gastrectomy. Its clinical presentations and management strategies in old patients have not been well described in available literature. This report describes the clinical features, management strategies, and treatment outcome of acute cholecystitis immediately after gastrectomy. Acute cholecystitis immediately after gastrectomy in old patients has different clinical presentations, such as fever and high plasma C-reaction protein level. Abdominal computed tomography (CT) scan and ultrasonography showed acute cholecystitis in our cases, which was treated with antibiotics and ultrasound-guided percutaneous transhepatic gallbladder drainage (PTGD). The results indicate that abdominal CT scan and ultrasonography can effectively diagnose acute cholecystitis after gastrectomy, which can be effectively treated with antibiotics and PTGD.

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Key words: Cholecystitis; Ultrasonography; Postoperative complication; Gastrectomy

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INTRODUCTION

Radical gastrectomy is the main procedure for advanced gastric carcinoma. Acute cholecystitis following radical gastrectomy has been rarely reported[1], although bile stasis and gallbladder ischemia may occur after vagotomy and gastrohepatic ligament dissection[2]. Compared with acute cholecystitis in the general population, acute cholecystitis immediately after radical gastrectomy, a clinical situation not well-recognized in old patients, is characterized by a delayed diagnosis and a high mortality rate. In this paper, we report its clinical features, management strategies, and treatment outcomes of 3 old patients.

CASE REPORT

Case 1

A 64-year-old Chinese female patient presented with upper abdominal discomfort and a 10 kg weight loss for 10 mo. Gastroscopy revealed an ulcerated tumor at the gastric antrum. Biopsy demonstrated a poorly- differentiated adenocarcinoma with no metastasis. This patient had no history of gallstones and cholecystitis. Preoperative computed tomography (CT) displayed a normal gallbladder (Figure 1A). Six days after distal gastrectomy, D_2 lymphadenectomy and Billroth Ⅱ reconstruction, she received enteral nutrition via a transnasal jejunal feeding tube. The patient had slight right abdominal and left shoulder pain but no fever and chilly on day 9 after operation. Physical examination showed right upper quad-



Figure 1 Computed tomography (CT) scan showing normal gallbladder before operation (A), thickened gallbladder wall and biliary sludge deposition on day 12 after operation (B), and normal gallbladder wall 7 mo after operation (C).

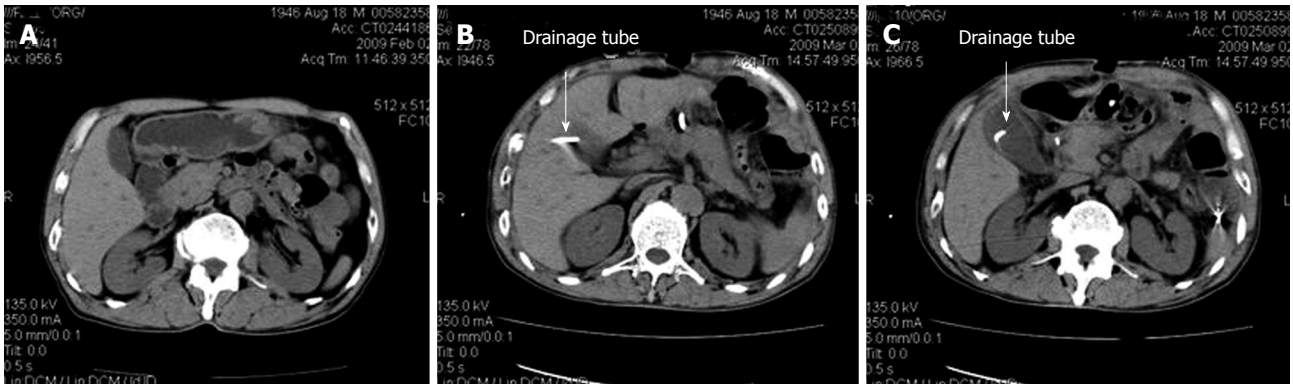


Figure 2 CT scan displaying normal gallbladder before operation (A), thickened gallbladder wall on day 16 after operation (B, C).

rant abdominal tenderness and muscle tension, but no rebound tenderness and Murphy's sign. Laboratory test showed normal white blood cells (WBC) and neutrophils in peripheral blood, as well as normal amylase level and liver function. Abdominal ultrasound and CT scan on day 12 after operation demonstrated thickened gallbladder wall and biliary sludge deposition (Figure 1B). She was given intravenous antibiotics on day 12 after operation for 24 d. Her symptoms disappeared on day 24 after treatment with antibiotics. The patient was discharged 50 d after gastrectomy. Abdominal CT scan displayed a normal gallbladder (Figure 1C) without abdominal pain 7 mo after gastrectomy.

Case 2

A 76-year-old Chinese male patient presented with melena for 6 mo. Gastroscopy showed an ulcerated tumor at the gastric antrum and biopsy demonstrated a moderately-differentiated adenocarcinoma with no metastasis. The patient had no history of gallstones and cholecystitis. Preoperative CT scan displayed a normal gallbladder (Figure 2A). Four days after distal gastrectomy, D_2 lymphadenectomy and Billroth Ⅱ reconstruction, he received enteral nutrition via a transnasal jejunal feeding tube. The patient developed high fever (39.4℃) and septic shock without abdominal pain on day 14 after operation. His plasma C-reaction protein (CRP) level was increased to 82.3 mg/L with normal WBC and neutrophils in peripheral blood as well as normal amylase level and liver function. Peripheral blood culture was positive for *Escherichia coli*. He received intravenous antibiotics on day 14 after operation for 6 d, but his body temperature did not become normal. Abdominal BUS and CT scan on day 15 after operation revealed thickened rough gallbladder wall (Figure 2B), and bile culture demonstrated *Escherichia coli*. Three days after ultrasound-guided percutaneous transhepatic gallbladder drainage (PTGD), his temperature returned to normal. He was discharged 30 d after gastrectomy.

Case 3

A 64-year-old Chinese male patient presented with abdominal pain for 1 year. Gastroscopy showed a cardiac ulcerated tumor and biopsy demonstrated a poorly-differentiated adenocarcinoma with no metastasis. The patient had no history of gallstones and cholecystitis. Preoperative CT and BUS displayed a normal gallbladder. Four days after total gastrectomy, D_2 lymphadenectomy and Roux-en-Y reconstruction, the patient received enteral nutrition via a transnasal jejunal feeding tube and developed severe right upper abdominal pain on day 14 after operation. Physical examination showed severe right upper abdominal tenderness. His body temperature was 38.8℃ and plasma C-reaction protein (CRP) level was increased

to 57.6 mg/L. The number of WBC in peripheral blood was 10.4 × 10^9/L with 82.1% of neutrophils. Abdominal BUS demonstrated thickened gallbladder wall with biliary sludge deposition on day 14 after operation and he underwent ultrasound-guided PTGD. Bile culture demonstrated *Escherichia coli*. His symptoms disappeared and body temperature decreased to 37.6℃ on day 15 after operation. He was discharged 22 d after gastrectomy.

DISCUSSION

Acute cholecystitis immediately after radical gastrectomy has been rarely reported[3-5]. Although its pathogenesis is still unclear, it may result from ischemia of gallbladder and biliary stasis, impaired contractile motility of gallbladder after gastrectomy and vagotomy, digestive tract reconstruction, and long-time preoperative fasting[2-4]. Its clinical features are inconsistent with those of acute cholecystitis in general population. Since the symptoms of acute cholecystitis in old patients appear on days 9-14 after gastroectomy, its diagnosis is often delayed and physical findings may be confused with other postoperative complications, such as duodenal stump leakage, postanastomotic leakage, abdominal cavity infection and incisional discomfort. Acute cholecystitis immediately after radical gastrectomy differs significantly from that in general population. The majority of old acute cholecystitis patients have right upper quadrant pain, fever, leukocytosis, and high plasma CRP. Severe bacterial infection may be an exclusive sign of acute cholecystitis immediately after radical gastrectomy in old patients. Case 2 had bacteremia and septic shock but no abdominal pain and tenderness. Abdominal CT and BUS scan confirmed acute cholecystitis immediately after radical gastrectomy by revealing a thickened gallbladder wall, gallstones or gallbladder sludge, pericholecystic fluid and inflammation. In case 1, acute cholecystitis immediately after radical gastrectomy was confused with incisional discomfort, which was diagnosed by abdominal CT and BUS scan on day 3 after operation. It was reported that cholecystectomy, cholecystostomy, ultrasound-guided percutaneous drainage or percutaneous transhepatic gallbladder drainage are the treatment modalities for acute cholecystitis immediately after radical gastrectomy. However, these treatment modalities are still controversial because their efficiency and safety have not been well evaluated. The mortality rate of patients with acute cholecystitis immediately after radical gastrectomy is over 20%[1]. According to our experience with the 3 cases, ultrasound-guided PTGD should be considered for patients with acute cholecystitis immediately after gastrectomy. When physical findings and/or laboratory test reveal postoperative infection, abdominal CT and BUS scan should be performed to diagnose acute cholecystitis immediately after gastrectomy. Ultrasoun-guided PTGD, as a minimal invasive surgical approach, is an efficient and safe method for acute cholecystitis immediately after gastrectomy in old patients.

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Single incision laparoscopic surgery

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Abstract

As a complement to standard laparoscopic surgery and a safe alternative to natural orifice transluminal endoscopic surgery, single incision laparoscopic surgery is gaining popularity. There are expensive ports, disposable hand instruments and flexible endoscopes that have been suggested to do this surgery and would increase the cost of operation. For a simple surgery like laparoscopic cholecystectomy, these extras are not needed and the surgery can be performed using standard ports, instruments and telescopes. Triangular port insertion and use of instruments by the "chop stick method" are recommended to successfully do the procedure as we have done in our so far small series of 40 cases.

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Key words: Single incision laparoscopic surgery; Cholecystectomy

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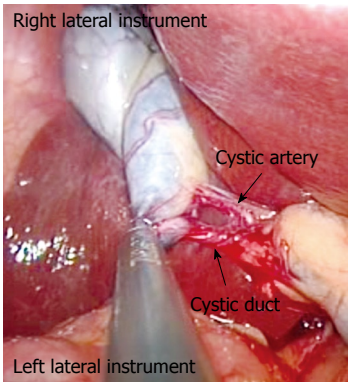


Figure 1 Instrument position while Calot's triangle is defined.

TO THE EDITOR

I read with great interest the article by Hirano *et al*[1] published in issue 2, volume 16 of *World J Gastroenterol* 2010. The use of loop is a good method for retraction without perforating the gall bladder. The method would increase safety of the procedure, but in purist terms, makes it a double incision laparoscopy (DILS).

We have done 40 single incision laparoscopic surgeries using conventional trocars, instruments and telescope. This is to avoid the increase in cost that is inevitable if we were to use two reticulating instruments and the more expensive semi flexible laparoscope or special ports[2].

By inserting the trocars in a triangular manner with a 10 mm telescope inserted from the top port, we are able to reduce sword fighting of instruments. The lower 5 mm ports are manipulated by the "chop stick method" developed by us to ensure that they do not clash. A 30° telescope, 5 mm in diameter, is introduced from the lower left cannula at the time when the 10 mm clip applier is introduced from the top cannula. The clips are only applied when a large window is made in the Calot's triangle (Figure 1).

After the first 20 cases, the instrument clashing, air leak,

and duration of surgery have reduced. This goes to show that there is a learning curve in this procedure[3] during which the main problems are of instrument clash and air leak.

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Meetings

Events Calendar 2010

January 25-26
Tamilnadu, India
International Conference on Medical Negligence and Litigation in Medical Practice

January 25-29
Waikoloa, HI, United States
Selected Topics in Internal Medicine

January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology Conference

January 28-30
Hong Kong, China
The 1st International Congress on Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of Gastroenterology & Endoscopy Meeting

March 09-12
Brussels, Belgium
30th International Symposium on Intensive Care and Emergency Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian National Association for Study of the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on Diabetes PACD14

March 25-28
Beijing, China
The 20th Conference of the Asian Pacific Association for the Study of the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology and Hepatology Conference, EGHC 2010

April 14-17
Landover, Maryland, United States
12th World Congress of Endoscopic Surgery

April 14-18
Vienna, Austria
The International Liver Congress™ 2010

April 28-May 01
Dubrovnik, Croatia
3rd Central European Congress of surgery and the 5th Croatian Congress of Surgery

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual Meeting

May 06-08
Munich, Germany
The Power of Programming: International Conference on Developmental Origins of Health and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and Rectal Surgeons Annual Meeting

June 04-06
Chicago, IL, United States
American Society of Clinical Oncologists Annual Meeting

June 09-12
Singapore, Singapore
13th International Conference on Emergency Medicine

June 14
Kosice, Slovakia
Gastro-intestinal Models in the Research of Probiotics and Prebiotics-Scientific Symposium

June 16-19
Hong Kong, China
ILTS: International Liver Transplantation Society ILTS Annual International Congress

June 20-23
Mannheim, Germany
16th World Congress for Bronchoesophagology-WCBE

June 25-29
Orlando, FL, United States
70th ADA Diabetes Scientific Sessions

August 28-31
Boston, Massachusetts, United States
10th OESO World Congress on Diseases of the Oesophagus 2010

September 10-12
Montreal, Canada
International Liver Association's Fourth Annual Conference

September 11-12
La Jolla, CA, United States
New Advances in Inflammatory Bowel Disease

September 12-15
Boston, MA, United States
ICAAC: Interscience Conference on Antimicrobial Agents and Chemotherapy Annual Meeting

September 16-18
Prague, Czech Republic
Prague Hepatology Meeting 2010

September 23-26
Prague, Czech Republic
The 1st World Congress on Controversies in Gastroenterology & Liver Diseases

October 07-09
Belgrade, Serbia
The 7th Biannual International Symposium of Society of Coloproctology

October 15-20
San Antonio, TX, United States
ACG 2010: American College of Gastroenterology Annual Scienitfic Meeting

October 23-27
Barcelona, Spain
18th United European Gastroenterology Week

October 29-November 02
Boston, Massachusetts, United States
The Liver Meeting® 2010--AASLD's 61st Annual Meeting

November 13-14
San Francisco, CA, United States
Case-Based Approach to the Management of Inflammatory Bowel Disease

December 02-04
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Acknowledgments

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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

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/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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Write as mean ± SD or mean ± SE.

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EDITORIAL

WJG sets an example of internationalization for other Chinese academic journals

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Abstract

Supported by the "Special Fund for Key Academic Journals" of the National Natural Science Foundation of China, *World Journal of Gastroenterology* (*WJG*) has become a high-impact international clinical medical journal due to the great efforts of Professor Lian-Sheng Ma, Editor-in-Chief, and his team over several years. Now, *WJG* has successfully achieved a high degree of internationalization and sets a good example for other Chinese academic journals.

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Key words: China; Academic journals; Internationalization

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CREATIVE PEOPLE DESERVE APPLAUSE

During the application period for the "Special Fund for Key Academic Journals" of the National Natural Science Foundation of China (NSFC) in early 2002, I came to know Professor Lian-Sheng Ma and *World Journal of Gastroenterology* (*WJG*), which he created. Since then, I have had close contact with him. As a successful entrepreneur and pharmacologist, he has devoted himself to the creation of academic journals by applying his past successful experience in the pharmaceutical industry to the academic publishing field. This shift carried a great personal risk but it has worked out well for the Chinese academic publishing industry. Professor Ma is always willing to listen to and incorporate other people's opinions and suggestions, and can thereby, provide creative ideas. He likes making friends and therefore keeps close contact with a number of government employees, scientists and publishers. During regular telephone or face-to-face conversations, Professor Ma often pushes me to give an in-depth opinion on the feasibility of his ideas. When *WJG* was initially supported by the NSFC "Special Fund for Key Academic Journals" in 2002, it was published bimonthly. Within a few years, *WJG* has become a weekly journal. Such rapid development has far exceeded my expectations and is a marvel in the Chinese academic publishing industry. For this reason, I have full confidence in the rapid development of Chinese academic journals in the next few years.

According to the traditional view, the basic conditions for Professor Ma to create international English-language medical journals are not very good. However, he is very good at exploiting the strengths of the journals to compensate for their inadequacies. For this reason, he can adeptly manage the overall operation of the journal and perform better than many other scientists and pub-

lishers. More importantly, he is not merely satisfied with issuing orders for the management of the journal operation. Instead, he always becomes personally involved in specific affairs, and therefore, has total control over the journal. He always participates in every aspect of journal production, whether the editing process or the design of the online version, constantly poses questions and suggests modifications, and immediately takes action. He not only has goals and ideas but also can find solutions to any problems that are encountered. These aspects constitute the basis for his success.

I was particularly touched by what Professor Ma did during the summer of 2003 while the severe acute respiratory syndrome epidemic was raging in Beijing. While many institutions came to a semi-standstill, Professor Ma launched the online version of *WJG* during that period. The overall outlook of online *WJG* is impressive and comparable to that of some world-renowned journals. At the beginning, thousands of visitors came to the site per day. Now, this figure has grown to tens of thousands, and *WJG* has become a leader among Chinese academic journals. Professor Ma's work style and creative spirit have rarely been seen in traditional Chinese publishers, because he has closely linked his personal interests (economic interests and social image) to the development of *WJG*.

The success of *WJG* has brought a fresh outlook to the Chinese academic publishing industry. Professor Ma should feel proud of his contributions, which have been made at a time when China is still not open to publishing privately funded academic journals. I hope that more successful and courageous Chinese entrepreneurs will become involved in the Chinese academic publishing industry; an area that remains to be developed, and one that can provide tremendous opportunities.

A JOURNAL REFLECTS A TYPE OF CULTURE

A journal not only reflects the experience and background of its editors/publishers, but also displays their unique personality and personal and cultural tastes. The readers can discover something beyond the contents of a journal. I have a habit of browsing world-renowned journals to capture changes in the overall trend and style of mainstream journals. I also look through *WJG*, from which I can glimpse inside Professor Ma's inner world and mind, and appreciate his efforts towards realizing his ambition to make *WJG* an excellent international journal.

With China's accession to the World Trade Organization and continuous rapid economic growth, its international status has been greatly improved. In recent years, a large number of foreign publishers and distributors have set up representative offices in China or have established cooperation with Chinese printers or publishers. I have had contact with more than 10 foreign publishers and therefore understand their primary intention and purpose. On many occasions, I have warned the publishers of journals supported by the NSFC Special Fund for Key Academic Journals to develop such cooperation with prudence. *WJG* Press was not the first domestic publisher to establish cooperation with well-known foreign publishers, but it was the first that dared to discontinue such cooperation. In this way, Professor Ma seeks internationalization of *WJG* not to further his own reputation, but rather that of the journal.

The development of *WJG* over these years has not been smooth, and has had many difficulties. We have always been concerned about the overall situation of NSFC-funded journals, especially those like *WJG* that have incurred much controversy. Between 2004 and 2005, the self-citation rate of *WJG* was 20%, which exceeded the ISI standard, and this resulted in *WJG* being removed from Science Citation Index (SCI). To combat these arguments, Professor Zuo-Yan Zhu, the NSFC Vice President, pointed out at a meeting in January 2003 that the goal of Chinese academic journals should be to achieve internationalization, to be included in SCI, and to be a leader among international journals in the same field. There are many ways to achieve these goals. However, the most fundamental way is to improve the quality of the journals. Although many approaches are available to improve the impact factor of a journal in a short period, there is no doubt that a high-impact journal can be created only after its scientific quality is raised.

As mentioned above, in 2004, *WJG* drew wide criticism because of a high self-citation rate. I sensed that Professor Ma underwent tremendous pressure during that period. However, it is pleasing to know that he did not make any excuses or avoid problems, but spent a lot of time to establish the reasons for this and make improvements. Fortunately, these lessons have led to the subsequent rapid development of *WJG*.

Ms. Emilie Marcus, Editor-in-Chief of *Cell*, pointed out in a forum in 2006 that the presence of high-impact articles depends on strong inputs of financial and human resources. These words are easier said than done. How is financial investment achieved? Where should the funds be invested? How are talented scientists recruited and utilized? Coincidentally, in recent years, *WJG* has been practicing the above sentiments. This shows that great minds think alike.

Looking back on modern technological developments worldwide, it can be seen that the progress of all renowned journals has not been smooth, but full of ups and downs. To create a world-renowned journal, the sponsor has to spend a lot of time and effort, sometimes even for several generations. Such journals are more respected and their publishing model is worthy of being passed on to others. The history of *Science*, which was created by Thomas Edison, spans 125 years, while that of British *Nature* and *The Lancet* (created by the Wakely family) spans 137 and 183 years, respectively. Although these journals are not run by governments, they enjoy a great reputation worldwide. I believe that Professor Ma could have the confidence and courage to make *WJG* a world-renowned journal.

PRACTICAL EXPERIENCE OBTAINED DURING *WJG* DEVELOPMENT HAS GREAT SIGNIFICANCE

At the last meeting of the *WJG* working group, I mentioned that the reform of state-owned enterprises could provide a reference for the successful operation of academic journals. Here, I would like to discuss the implications of the rise of the Chinese Communist Party (CCP) for the reform of Chinese academic journals.

In recent years, many entrepreneurs in China have been keen to read about the developmental history of the CCP, to gain a better insight into business management. As the world's largest political party (with nearly 70 million members), the CCP has been in existence for 85 years and has ruled China for more than half a century. What are the fundamental reasons for its sustainability? This question has attracted the interest of many domestic and foreign politicians and entrepreneurs. The rapid growth of China's national economy since 1979 has led state-owned enterprises into a new era of development. All enterprises are in competition with their rivals for the throne. The rise of new outstanding enterprises is often coupled with the fall of some previously successful companies. The rapid turnover of businesses has become a feature of the development of the Chinese economy. Survival is the main theme of business management in our times.

Ten years ago, entrepreneurs might have been successful merely by having the courage to go into business. In more recent times, however, the survival of enterprises has been more dependent on management rather than luck or back-door dealing. For this reason, entrepreneurs have been anxious to seek better management approaches: some have learned from the West, some from Confucianism, and others from the developmental history of the CCP.

Western management style has its own advantages, but it may not be suitable for Chinese enterprises. Although Chinese entrepreneurs hope to derive the essence from Western management style, they tend to spend a lot of time dealing with superficial matters. The Western business community has a history of 200 years and is very mature. Many excellent Western academic journals also date back for more than a century. A mature business environment provides Western academic journals with a sound management system. In contrast, the development of Chinese academic journals has occurred mainly after 1976. As a result of market, cultural and policy differences, there is no appropriate management system available for Chinese academic journals. This developmental stage provides a better opportunity for publishers to display their talents.

In terms of business management, an important criterion for entrepreneurs to judge the feasibility of a management theory is whether it is practical (in relation to the situation in China). The core issue with which entrepreneurs are concerned is how to realize Western management style in China. The ultimate goal of businesses is to achieve a profit, therefore, they advocate the same laws as the CCP: survival of the fittest and natural selection. How to guarantee basic living conditions is their common primary goal in response to external challenges. The development of enterprise management has about 100 years of history. As a result, various types of business management theories have been proposed. Despite diversity, their fundamental purpose is to answer the same question: how is efficiency improved? The CCP can organize many citizens in a fairly orderly manner and has an extremely high level of organization that is universally acknowledged. High efficiency and strong executive power are its biggest features.

The operation of academic journals has its own laws. If an academic journal is not run in accordance with market or economic rules, but managed in a very superficial manner, the journal will not be successful. *WJG* is aiming to implement Western management style in China by obeying market and economic rules. The success of *WJG* will make a significant contribution to Chinese scientific and cultural development. I hope Professor Ma can keep a diary to record his management practice, which could be a great asset to later generations.

Our generation, which is involved in the reform of China's academic journals, should not overlook the development of *WJG*, because its preliminary success has been achieved by overcoming many obstacles and fighting against the inherent snobbishness of the traditional publishing industry that has existed in China for decades. I am very pleased that some of my ideas for creating outstanding international academic journals have been adopted and gradually realized by Professor Ma. I am also happy that *WJG* sets an example of internationalization for other Chinese academic journals.

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EDITORIAL

Spices, herbal xenobiotics and the stomach: Friends or foes?

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Abstract

Spices and herbal remedies have been used since ancient times to treat a variety of disorders. It has been experimentally demonstrated that spices, herbs, and their extracts possess antimicrobial, anti-inflammatory, antirheumatic, lipid-lowering, hepatoprotective, nephroprotective, antimutagenic and anticancer activities, besides their gastroprotective and anti-ulcer activities. Despite a number of reports on the toxicity of herbs and spices, they are generally accepted as safer alternatives to conventional therapy against gastric ulcers. To this end, it is also believed, that excessive consumption of spices may favor the pathogenesis of gastric and duodenal ulcer and some studies have substantiated this common perception. Based on various *in vivo* experiments and clinical studies, on the effects of spices and herbs on gastric ulcers, it has indeed been shown that certain spices do possess remarkable anti-ulcer properties mediated by antisecretory, cytoprotective, antioxidant, and anti-*Helicobacter pylori* effects and mechanisms regulated by nitric oxide, prostaglandins, non-protein sulfhydryl molecules and epidermal growth factor expression. Accordingly, their consumption may attenuate and help prevent peptic ulcer disease. In the present review, the beneficial effects of spices and herbal nutritive components on the gastric mucosa are discussed against the paradigm of their deleterious potential.

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Key words: Herbs; Spices; Stomach; Stomach ulcers; Anti-ulcer agents

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INTRODUCTION

Herbs and spices are considered as an important additive to food in several parts of the world. They enhance aroma, piquancy, and impart flavor to food. For long, they have been regarded as the essential component of eastern cuisine and have also been adopted into western diets. Since ancient times, spices and herbs have also been used in traditional treatment of a number of diseases. Nowadays, several experimental studies and, to a lesser extent, clinical trials have also emphasized the role of herbs in the treatment of a variety of disorders[1-4]. Several herbs and herbal extracts have been shown to possess antibacterial properties[5-7]. For instance, onion, garlic, ginger, pepper and mustard have demonstrated antimicrobial activity against several types of bacteria[8]. Tayel and El-Tras have recently reported a potent antibacterial activity of cinnamon and clove against several bacterial strains[9]. Some spices possess antifungal activity[10,11]. Beside their antifungal activity, herbs have also shown vermicidal, nematocidal and molluscicidal potential[12-15]. In addition, gingerol, the active ingredient of ginger, and eugenol have shown anti-inflammatory

and antirheumatic activity[16]. More recent studies have also demonstrated anti-inflammatory and antirheumatic properties of herbs[17-19]. Furthermore, gingerol and curcumin have also shown lipid-lowering potential in experimental animals[20-22] as well as in clinical trials[23,24].

In addition, many studies have especially endorsed the experimental evidence of folkloric utilization of spices to treat gastrointestinal disorders, without gastric mucosal toxicity. Also clinical, video-endoscopic studies conducted by Graham *et al*[25] have shown an adaptive cytoprotection. Furthermore, several experimental studies have also demonstrated cytoprotective activity of herbs[26-29].

On the other hand, some spices are considered to be toxic to the gastric mucosa and may potentiate or induce gastric injuries. Gastric mucosal toxicity induced by some spices in experimental animals is related to their oxidative constituents such as phenylpropenes, safroles, methyl eugenol, 1'-hydroxyestragole, myristicin and elemicin[30,31]. Meyers *et al*[32] have reported deleterious effect of red pepper and black pepper on the stomach. Both have induced a significant enhancement in parietal secretion, pepsin secretion, and potassium loss, as well as a dose-dependent gastric cell exfoliation and mucosal microbleeding, which are comparable to those induced by aspirin. Other studies have also reported epigastric pain and dyspepsia. The mechanism of epigastric pain and dyspepsia induced by red and black pepper is not well-defined. However, it is believed to be a consequence of inhibition of gastric surface hydrophobicity, enhancement of surface wettability and activation of intramucosal pain receptors[33]. Some spices may stimulate acid secretion and have deleterious effects on the gastric mucosal lining. Intragastric perfusion of albino rats with aqueous extracts of red pepper, fennel, omum/ajwain, cardamom, black pepper, cumin and coriander have stimulated a cholinergic response, and/or *via* other mechanism(s) have induced acid secretion with a respectively declining order. In injured stomach, cumin and coriander increase gastric secretion, and red pepper has an inhibitory effect[34].

Herbs in general are believed to be safe, and several studies have found them to be non-detrimental and beneficial to gastric mucosa and have cytoprotective properties[25,28]. In addition to their anti-ulcerogenic activity, it has been reported that spices contain protective factors that possess anticancer potential[35-37]. The ulcerogenic and/or anti-ulcerogenic activity of herbs is likely to be due to the oxidative and anti oxidative action of their different phytoconstituents. The prominent mechanism of protective action is mediated by their antioxidant activity and the ability to scavenge reactive oxygen species (ROS). A variety of herbs such as clove, cinnamon, oregano, black pepper, turmeric, ginger and polygonum species are known to contain phytoconstituents with anti-oxidative potential[38-46]. Antioxidative constituents of herbs are summarized in the Table 1.

The beneficial effects of herbs and plant extracts in the prevention of gastric injury have been indicated in several experimental studies. For example, various extracts of *Mammea americana* have significantly reduced the ulcer index and demonstrated a cytoprotective effect in experimentally induced ulcers[47]. Further pharmacological and histopathological studies have demonstrated significant protection against ethanol-induced ulcers in rats by extracts of roots of *Asphodelus aestivus* and *Cichorium intybus*, herbs of *Equisetum palustre* and *Viscum album ssp.* album and fruits of *Laurus*[48].

Clinical studies have also confirmed the gastric protection conferred by herbs. In a clinical study of 98 outpatients with chronic gastritis, who were randomly divided into a group treated with herbal pairs and a control group treated with *Banxia Xiexin Tang*, effectiveness was significantly higher in the treated group. The treatment has improved therapeutic effects, and minimized the adverse effects of gastritis[49]. In another clinical study, 103 patients with duodenal ulcer received phytotherapy in the form of infusions and concoctions of medicinal plants. This resulted in ulcer scarring and a decrease in relapse[50]. Furthermore, in a study of 170 patients with duodenal ulcer and gastroduodenitis treated with herbal combinations alone or with addition of antacids, pain and dyspeptic symptoms disappeared in > 85% of both treatment groups, with a similar rate of endoscopic healing[51]. Hence, agents that possess antioxidative activity are expected to play a major role in the treatment of peptic ulcer disease.

EXPERIMENTAL INDUCTION OF ULCERS

Various noxious chemical agents are used to induce acute experimental gastric ulceration. Indomethacin and necrotizing agents including 80% ethanol, 0.2 mol/L NaOH, 25% NaCl are commonly used. Pyloric ligation is applied in antisecretory studies[52-56]. Other universally accepted experimental ulcer models include stress induced by swimming[55], aspirin[56], ethanol/HCl, acetylsalicylic acid, cold-restraint[57] and hypothermic restraint[54]. In addition, gastric ulcers have also been induced by serosal application of acetic acid[58].

FACTORS INVOLVED IN ULCER HEALING

Acid and other noxious agents such as bile acids, non-steroidal anti-inflammatory drugs (NSAIDs) and ethanol enhance the presence of mucosal barrier disruption, H^+ back diffusion and ulcer susceptibility. Adequate mucosal flow and secretion of bicarbonate with formation of an alkaline buffer layer at the epithelial surface, is considered as a first line of mucosal defense. Prostaglandins (PGs) are involved in the ulcer healing process. Growth factors, some gut hormones (e.g. gastrin and cholecystokinin) and melatonin promote ulcer healing through generation of cyclooxygenase-2 (COX-2) and release of PGE2 in the ulcer margin[59,60]. Similar action is achieved by application of antisecretory doses of exogenous PG[61]. In addition to PGs, many other factors including growth factors, nitric oxide or calcitonin gene-related peptide, as well as some gut hormones such as gastrin and cholecystokinin, leptin,

Table 1 Antioxidant constituents of herbs

Antioxidant constituents	Herb	Ref.
Pimentol, biflorin	Clove, allspice	[38]
Phenolic constituents: phenolic acids, phenolic diterpenes, flavonoids, and volatile oils, phenolic volatile oils	Many herbs including clove, cinnamon and oregano	[39]
Piperine	Black pepper	[40]
Curcumin, methoxy phenols, dehydrogingerdione, bakuchiol	Turmeric, from Indian spices, ginger, *Psoralea corylifolia*	[41]
Phenolic compounds: flavonoids, phenolic acids and their derivatives, tannins, stilbenes, and anthraquinones	Polygonum species, spring onion, broccoli, orange, carrot and ginger	[42]
Phenolic diterpenes	*Rosmarinus officinalis* and *Salvia officinalis*	[43]
Phenolic, flavonoid, chlorogenic acid and neochlorogenic acid	Andean spice Sanicula graveolens (Apiaceae)	[44]
Phenylpropanoids (phenolic compounds)	Human diet, spices, aromas, essential oils, propolis and traditional medicine	[45]
Polyphenolic	*Piper umbellatum, Piper nigrum, Piper guineense*	[46]

ghrelin and gastrin-releasing peptide, are involved in gastroprotection. The protective action of gut hormones has been attributed to the release of PG or activation of sensory nerves[62].

MECHANISMS OF PROTECTION RENDERED BY SPICES AND HERBS

The mechanism of herb-induced gastroprotection varies according to the nature and chemical constituents of the herbs. Three main functions including antisecretory, cytoprotective and antioxidant activities, isolated or in combination, are responsible for gastric mucosal protection.

Antisecretory activity

Several herbs and plants extracts have shown an anti-ulcer effect mainly due to their potent antisecretory action, which could be related to their flavonoid content. In experimental animals, *Cissus quadrangularis* extract and *Maytenus ilicifolia* have been shown to inhibit gastric secretions and ulcer index[63,64]. Similarly, phytosphingosine hydrochloride has been shown to have antisecretory and anti-ulcer effects in animals with pyloric ligation[65]. Methanolic extract of *Momordica charantia* prevents development of peptic ulcer and promotes healing of ulcers induced by acetic acid. Antisecretory studies in pylorus-ligated rats have demonstrated a significant reduction in acidity, pepsin content and ulcer index. On the other hand, the extract also amplifies the gastric mucosal content[66]. A variety of herbs and plant extracts including standardized aqueous extract of *Cecropia glaziovii Sneth* (Cecropiaceae)[67], alkaloid extract and 2-phenylquinoline obtained from the bark of *Galipea longiflora* (*Rutaceae*)[68] and *Landolphia owariensis* extracts[69] have exhibited an anti-ulcer effect.

The inhibition of gastric acid and pepsin output in rats with intact or deactivated sensory nerves treated parenterally with capsaicin could contribute to the capsaicin-induced gastroprotection against acid-dependent mucosal lesions[70].

Cytoprotective activity

In pylorus-ligated rats, methanolic extract of *M. charantia L.* fruit protects against peptic ulcer and promotes ulcer healing *via* enhancing the gastric mucosal content and antisecretory activity in stress-, ethanol-, indomethacin- and cysteamine-induced ulcers[66]. In both ethanol- and indomethacin-induced experimental ulcers, pretreatment with isopulegol, a monoterpene present in essential oils of several aromatic plants, has resulted in significant gastroprotective action, which is apparently mediated through its endogenous PGs and antioxidative properties[71]. Moreover, Weikang (WK) decoction significantly protects against ethanol-induced gastric mucosal injury. This activity is mediated by enhancement of epidermal growth factor (EGF) content in gastric juice, nitric oxide (NO) in gastric tissue, PGE2 and superoxide dismutase (SOD) in plasma, inhibition of malondialdehyde (MDA) and endothelin in plasma, and an increase in mucosal thickness[72]. In swim- and ethanol-stress-induced ulcers, extract of ginger rhizome (*Zingiber officinale*) normalizes antioxidant enzymes and protects against oxidative and gastric mucin damage[55]. The gastroprotective effect of *Vanillosmopsis arborea* bark essential oil is likely mediated by α2-receptor activation[73], whereas the anti-ulcerogenic effect of *Solanum torvum Swartz* (Solanaceae) aqueous and methanol extracts is probably due to cytoprotective mechanisms[74]. Cytoprotective action of total carotenoid and astaxanthin esters depends on their mucin-related protective action as well as enhancing antioxidants enzymes level and H^+, K^+-ATPase inhibitory activity[75]. Synthesis of cytoprotective PGs, increased resistance of gastric mucosa, and inhibition of leukotriene synthesis are possibly responsible for gastroprotection induced by boswellic acids (from *Boswellia*)[57].

In various ulcer models in mice, the gastroprotective activity of *G. longiflora* (Rutaceae) is related to an increase in gastric mucus content and antisecretory activity. Additionally, NO is involved in mucosal protection, which could be attributed to their alkaloids, particularly 2-phenylquinoline[68]. Pretreatment of albino rats with aqueous extract orally once daily for 2 wk significantly inhibited HCl/ethanol-induced ulcers and enhanced gastric mucus production. Additionally, it displayed antisecretory activity in pylorus-ligated rats. The results indicate that the leaf extracts of *Landolphia* possess anti-ulcer properties[69].

A new flavonoid derivative, DA-6034, also prevents ulcers induced by ethanol, aspirin, indomethacin, stress, and acetic acid, and enhances endogenous PGE2 syn-

thesis and mucus content in the gel layer of the gastric mucosa. Promotion of the gastric defensive systems is the likely cause of its gastroprotective activities[56].

Antioxidative activity

Oxidants are implicated in the pathophysiology of various diseases, including peptic ulcer disease, and antioxidants may therefore contribute to their prevention and treatment. NSAIDs account for some of the most commonly used drugs worldwide and may activate the oxidative process. For instance, treatment with indomethacin induces neutrophil activation with release of ROS and microvascular injuries, which is considered as the prime event in gastric mucosal damage[76]. Therefore, investigators have continued to search for agents with antioxidative properties, which prevent or at least reduce ROS-induced mucosal damage. Such agents include isopulegol, which has shown significant gastroprotective effects in ethanol- and indomethacin-induced ulcer models. Gastroprotective action is probably mediated by its antioxidative properties, synthesis of endogenous PGs and K^+ (ATP) channel opening[71]. Other herbs with antioxidative actions include carotenoid and astaxanthin esters, the herb collection Korniozil, and ginger rhizome. For instance, total carotenoid and astaxanthin esters have experimentally protected gastric mucin and increased levels of the antioxidant enzymes catalase, SOD, and glutathione peroxidase in gastric homogenate[75]. In addition, Korniozil also protects against experimentally induced stress ulcers, with enhancement of gastric mucous coat regeneration, along with restoration of lipid peroxidation and antioxidative system function[77]. Ginger rhizome extract has shown gastroprotective activity in animals with swim- and ethanol-stress-induced ulcers. This activity is based on gastric mucin generation, restoration of antioxidant enzymes and inhibition of *Helicobacter pylori* (*H. pylori*) growth[55].

The gastroprotective effect of *Cissus sicyoides* extract administered orally in rodents treated with various ulcerogenic agents is also due to its antioxidative properties, increase of NO and SH groups, with enhancement of mucosal defense mechanisms and microcirculatory response. Therefore, the authors have supported its use for gastric ulcer treatment[78].

Studies on piperine have demonstrated an antioxidative effect *in vitro*, lipid peroxide inhibition *in vivo*, and enhancement of the bioavailability of phytochemicals. The protective activity of piperine is linked to its antioxidative action and ability to inhibit ROS[40]. Thymoquinone, the active constituent of *Nigella sativa*, has been shown to protect against gastric mucosal damage induced by ethanol. These effects can be ascribed to improvement in the antioxidant status, increased serum levels of serum glutathione, SOD, inhibition of radical oxygen species, and increased mucin content of the gastric mucosa[29,79].

Other activities: PG-herb interaction

PG synthesis is important for gastric mucosal protection. PG has been used as an antisecretory drug to treat peptic ulcer disease. However, the use of a sufficient therapeutic dose is associated with adverse effects, which limits its use, especially after the introduction of antisecretory drugs such as H2-receptor antagonists and proton pump inhibitors. Several studies on herbs and plant extracts that activate PG synthesis have emphasized their role in gastroprotection and healing of gastric mucosal injuries. Silva *et al*[71] have reported a significant dose-related protective effect of isopulegol, a monoterpene that is obtained from essential oils of several aromatic plants, against indomethacin- and ethanol-induced ulcers. This protective action is probably mediated by endogenous PGs, K^+ (ATP) channel opening, and antioxidant properties. Similarly, Alqasoumi *et al*[54] have reported a PG and/or antisecretory, as well as antioxidant-mediated gastroprotective activity of Rocket extract. Boswellic acid, a triterpenoid used clinically to treat arthritis, has exhibited a mucosal protective action through PG synthesis stimulation and leukotriene synthesis inhibition[57]. Endogenous PGs and PG EP1 receptors have an important role in the adaptive protection and functional responses in mice treated with sodium taurocholate[80]. The active ingredient of chilli, capsaicin, which is believed to be ulcerogenic, on the contrary, prevents ulcer formation through its antisecretory action, as well as stimulation of mucus, alkali secretions and mucosal microcirculation. In addition, capsaicin stimulates afferent neurons in the stomach and transmits signals to the central nervous system, which trigger an anti-inflammatory response and gastroprotection[81]. Treatment with small doses of topical capsaicin protects the gastric mucosa from damage by strong irritants[69].

PGs have been found to reduce enhanced duodenal mucosal permeability induced by hydrochloric acid[82]. In acute injury, small bowel mucosa function recovers with the restitution of epithelium, which is believed to be pivotal for epithelial repair, and occurs only in the presence of PG-mediated paracellular space closure[83]. Hatazawa *et al*[84] have studied the role of PGs/COX in the healing of indomethacin-induced small-intestinal ulcers in rats. They have reported ulcer healing by endogenous PGs, which is mediated by PG EP4 receptors, and involvement of COX-2 in the early stage and COX-1 in the late stage of healing. Bacterial lipopolysaccharide is involved in gastric mucosal protection in rats, *via* activation of COX and endogenous PG genes[85]. In rats, *Teucrium polium* has demonstrated protection against indomethacin-induced gastric ulcer through PG synthesis, EGF receptor (EGFR) expression and modulation of mucin secretion, besides its antioxidative activity, and it lack of toxicity[86].

EGF and effect of herbs

Ulcer preventive activity of herbs in experimentally induced gastric injury is thought to be mediated through their antisecretory and antioxidative properties, as well as PG generation. Various factors are involved in mucosal defense and repair, including gastrin, parietal cells and tumor necrosis factor (TNF)-α. Gastrin and parietal cells play an important role in the regulation of mucosal proliferation in response to gastric injury and inflammation[87]. Similarly, TNF-α, which is released during gastric mucosal

injury, also contributes to epithelial cell repair in the gastric mucosa *via* its receptor and activation of the PG pathway[88]. EGF and other growth factors are also pivotal for the process of mucosal healing. EGF and transforming growth factor (TGF)-α have a common receptor (EGFR). They promote ulcer healing through enhancement of cell proliferation, overexpression of growth factors, inhibition of gastric secretion and enhancement of blood flow at the ulcer margin[89]. Treatment with EGF significantly induces extracellular signal-regulated kinase (ERK) activity, COX-2 and PGE2 generation, and cell proliferation. The EGF-induced proliferation of gastric epithelial cells is probably mediated by the ERK/COX-2 pathway[90]. Gastric mucosa regeneration, with cell proliferation, migration, tissue injury repair and ulcer healing are controlled by activation of EGFR. Although TGF-α acts under normal circumstances and following acute injury, EGF acts mainly during the healing process of chronic ulcers. Both TGF-α and EGF, and other growth factors including basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), promote restoration of connective tissue and angiogenesis in injured gastric mucosa. Further growth factors involved in regeneration include keratinocyte growth factor, hepatocyte growth factor and trefoil peptides[91]. Regeneration of injured human gastric epithelial monolayers has been promoted by EGFR-dependent phosphoinositide 3-kinase activation[92].

Besides its role in gastric inflammation and injury, EGF plays an important role in the regulation of cancer growth. Phosphorylation of EGFR and inhibition of heparin-binding EGF-like growth factor (HB-EGF) carboxyl-terminal fragment (CTF), and HB-EGF-CTF nuclear translocation are considered crucial in inhibitory regulation of cancer cell growth[93]. Overexpression of EGF has often been found in gastric cancer. Growth factors alter the localization of tight-junction-associated proteins such as ZO-1 and occludin[94]. Baek *et al*[95] have also reported EGF overexpression and urokinase plasminogen activator receptor in human gastric cancers. In epithelial and mesenchymal cells, EGF binds to tyrosine kinase receptor and promotes malignant formation as well as tissue repair[96].

Herb-induced growth factor enhancement may support treatment strategies for gastric ulcer and cancer. Capsaicin-sensitive nerves contribute to healing of acetic-acid-induced chronic gastric ulcer through stimulation of EGF expression in salivary glands, serum and gastric mucosa[97]. Weitongning herb increases EGF and NO content in ulcer scars, which improves ulcer healing and reduces recurrence[98]. Also, in ethanol-induced gastric mucosal injury, WK decoction has shown a significant gastroprotective effect that is mediated by increased levels of NO in gastric tissue, PGI2 and SOD in plasma, and EGF in gastric juice[72]. Mexican tea herb and pilular adina herb have also demonstrated protection of gastric mucosa through stimulation of NO and EGF secretion and enhancement of EGFR expression[99]. Wang *et al*[100] have also reported increased expression of EGF and EGFR mRNA in experimental gastric ulcer in rats treated with Kuiyangping, with possible promotion of ulcer healing and decreased ulcer recurrence. *Angelica* and *Chuanxiong* spices given to rats with myocardial infarction may affect VEGF expression and promote endothelial cell proliferation[101]. This angiogenic effect may also be applied to the formation of new vessels in granulation tissue and restitution of injured gastric mucosa.

Through their effect on EGF, herbs may prove beneficial in the management of cancer. Constituents of ginger have inhibited EGF-induced cell transformation[102]. Inhibition of EGFR tyrosine kinase proliferation and invasion of gastric cancer cells has also been achieved by curcumin[103]. Molecular therapies that target growth factors EGF and VEGF and their receptors have shown promise against hepatocellular carcinoma in the absence of beneficial effects of chemotherapy[104]. Recent data combining EGFR and VEGF inhibitors have suggested the superiority of targeting multiple pathways rather than a single pathway[105]. The anticancer potential of curcumin has been demonstrated by inhibition of EGF-induced upregulation of aquaporin and ovarian cancer cell migration[106].

Role of NO mediation by herbs

Tsai *et al*[107] have evaluated the effect of some spices on NO overproduction generated by inducible NO synthase (iNOS), which is implicated in disease development. Rosemary, tarragon, oregano, basil, marjoram, allspice, and thyme have demonstrated poor to moderate activity. In contrast, cinnamon has excellent ability to scavenge NO. NO-scavenging activity of herbs is probably related to their high content of phenolic compounds, which scavenge NO or suppress iNOS. In another study, pretreatment with NOS inhibitor attenuated the gastroprotective effect induced by polyalthic acid[108]. Moreover, plant-extract-induced gastroprotection is probably related to the enhancing effect on NOS inhibitor expression, gastric microcirculation and release of NO[109]. Participation of NO, PGs and SH compounds may explain the anti-ulcerogenic action of diterpenoid from *Croton reflexifolius*[108].

Non-protein-SH compounds and herbal involvement

Non-protein (NP)-SH compounds contribute to gastric mucosal defense. Depletion of NP-SH alters mucosal integrity. *Commiphora opobalsamum* (L.) Engl. (Balessan) protects against various models of experimental gastric ulcers in rats. It has been shown to protect in a dose-dependent manner against mucus and NP-SH depletion with a large margin of safety[52]. Methanolic *C. sicyoides* extract given orally to rodents has been shown to inhibit experimental gastric injuries induced by necrotizing agents, *via* participation of NP-SH compounds and NO, and enhancement of the defense system[78]. Maintaining adequate gastric mucus concentration and NP-SH levels is essential for gastric mucosa integrity and function. Other herbs and plant extracts also have the ability to preserve the defense system of the gastric mucosa. For instance, pretreatment with *Ginkgo biloba* extract has been shown to inhibit, in a dose-dependent manner, ethanol-induced NP-SH compound production, depletion of gastric wall mucus con-

centration, and lipid peroxidation, and preserve mucosal function[110]. Similarly, anise aqueous suspension, and rocket and anise have significantly replenished ethanol-induced gastric wall mucus concentration and NP-SH depletion in experimental studies[54,111].

H. pylori bactericidal activity of herbs

The association between *H. pylori* and peptic ulcer disease is well-established and eradication is pivotal for ulcer healing and minimizing the relapse rate. Although the eradication rate of currently used regimens ranges between 80% and 90%, the problem of developing resistance is emerging. A number of investigators have evaluated the effect of herbs and plant extracts on *H. pylori*. Methylene chloride cinnamon extract has also shown an inhibitory effect on the free urease of *H. pylori*[112]. Also curcumin and its methanolic extract have inhibited the growth of all strains of *H. pylori in vitro*[113]. In addition, gingerol, a polyphenolic constitute of ginger root, has also demonstrated inhibitory activity on CagA+ strains of *H. pylori*[114]. Moreover, eugenol and cinnamaldehyde prevented growth of *H. pylori* obtained from human gastric tissue, and inhibited the growth of all 30 tested *H. pylori* strains, with a lack of resistance[115]. In a declining order, turmeric, cumin, ginger, chilli, borage, black caraway, oregano and liquorice have demonstrated partial bactericidal activity against *H. pylori*. *H. pylori* adhesion to the stomach has been inhibited by extracts of turmeric, borage and parsley[116]. Zaidi *et al*[117] have reported that > 50% of 50 commonly used Pakistani medicinal plants have inhibited the growth of eight *H. pylori* strains. *Curcuma amada* Roxb., *Mallotus philippines* (Lam) Muell., *Myrisctica fragrans* Houtt., and *Psoralea corylifolia* L aqueous-ethanol extracts have demonstrated a potent anti-*H. pylori* activity and *Mal. philippines* (Lam) Muell. has exhibited potent bactericidal activity.

SAFETY AND TOXICITY OF SPICES

The majority of experimental studies have reported a lack or low levels of toxicity for most spices. However, there are many case reports, and *in vivo* and *in vitro* toxicological studies that have demonstrated the toxicity of certain herbs and their constituents[118]. Some investigators have reported hepatotoxicity of curcumin and its derivatives[119], as well as turmeric and its ethanolic extract in vulnerable mice[120]. Longer treatment with high turmeric dose has been associated with a significant decline in body weight gain and alterations in liver weight[121]. In contrast, chronic treatment with *Foeniculum vulgare* ethanolic extracts of fruit and *Ruta chalepensis* aerial parts have resulted in a significant weight gain in male mice[122]. Changes in liver, spleen, lung or reproductive organs, along with a significant increase in sperm count and motility, and decreased hemoglobin have been reported in *Cinnamon zeylanicum*, *Piper longum* and *R. chalepensis*-treated animals[122,123]. In an experimental model, piperine (10 and 20 mg/kg) decreased mating performance and fertility. Five days post-mating, oral treatment induced considerable anti-implantation activity. In addition, intrauterine injection of piperine has caused loss of implants. However, piperine treatment has not produced any histopathological effect in the ovaries and uterus at the cellular level[124]. Although initial reports on the safety of black pepper and its active ingredient piperine were controversial, the current literature has established its safety in animals. Moreover, piperine has shown antimutagenic and antitumor activities. Curcumin administered at higher doses has resulted in nuclear, genome and more extensive mitochondrial damage in human hepatoma G2 cells, mediated by the elevated concentration of ROS and lipid peroxidation. Curcumin also has caused genotoxicity in PC12 cells and has induced suicidal death of normal erythrocytes[125-127]. At elevated concentrations, thymoquinone may be genotoxic and cytotoxic, induce programmed cell death in erythrocytes, and cause glutathione depletion and liver damage[128,129].

CONCLUSION

It continues to be debatable whether spices and herbal xenobiotics are beneficial to gastric mucosal damage, and whether their toxicity outweighs any benefits. It is, however, clear that the consumption of certain common spices in food and the intake of herbal supplements may help the fight against peptic ulcer disease in humans. This review provides insights into the future use of herb- and spice-based drugs as alternative treatments for gastric ulcer. Dietary and nutritional practices in the oriental region and the consumption of spices in moderate quantities may be of benefit for the prevention of gastric ulcer. The review suggests the need for further investigations into the benefit of herbal xenobiotics and spices on the gastric mucosa, with in-depth *in vivo* studies, sustained clinical trials and cohort analyses. There is enough evidence to conclude that spices have a beneficial effect on gastric ulcers, despite various reports of their toxicity.

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TOPIC HIGHLIGHT

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Treatment of iron deficiency anemia associated with gastrointestinal tract diseases

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Abstract

The gastrointestinal (GI) tract is a common site of bleeding that may lead to iron deficiency anemia (IDA). Treatment of IDA depends on severity and acuity of patients' signs and symptoms. While red blood cell transfusions may be required in hemodynamically unstable patients, transfusions should be avoided in chronically anemic patients due to their potential side effects and cost. Iron studies need to be performed after episodes of GI bleeding and stores need to be replenished before anemia develops. Oral iron preparations are efficacious but poorly tolerated due to non-absorbed iron-mediated GI side effects. However, oral iron dose may be reduced with no effect on its efficacy while decreasing side effects and patient discontinuation rates. Parenteral iron therapy replenishes iron stores quicker and is better tolerated than oral therapy. Serious hypersensitive reactions are very rare with new intravenous preparations. While data on worsening of inflammatory bowel disease (IBD) activity by oral iron therapy are not conclusive, parenteral iron therapy still seems to be advantageous in the treatment of IDA in patients with IBD, because oral iron may not be sufficient to overcome the chronic blood loss and GI side effects of oral iron which may mimic IBD exacerbation. Finally, we believe the choice of oral *vs* parenteral iron therapy in patients with IBD should primarily depend on acuity and severity of patients' signs and symptoms.

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Key words: Anemia; Inflammatory bowel disease; Intravenous iron; Iron deficiency; Oral iron

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INTRODUCTION

Iron deficiency anemia (IDA) is a consequence of depletion of body iron stores due to decreased iron uptake or increased iron loss/use. Body iron content is imposed by controlling its entrance into the body through the gastrointestinal (GI) system rather than by controlling its excretion. GI diseases are among the most common etiologies of IDA because the GI tract is a common site of blood loss and GI diseases may cause malabsorption of iron. Treatment of IDA may be especially cumbersome in patients with GI diseases due to relatively poor tolerability of oral iron preparations and decreased GI iron absorption. The goal of this review is to increase awareness of parenteral iron therapies in the treatment of IDA associated with GI diseases, particularly inflammatory bowel diseases (IBDs).

IRON DEFICIENCY ANEMIA

Clinical findings

Iron deficiency causes anemia through impaired heme synthesis in maturing erythrocyte precursors. In addition to typical symptoms of anemia, patients may present with pica (appetite for bizarre foods), atrophy of upper GI mucosa (glossitis, stomatitis, achlorhydria), and spooning of the nails (koilonychia). Achlorhydria may further worsen anemia by decreasing iron absorption through the GI tract. IDA is also associated with cervical esophageal webs (Plummer-Vinson syndrome), especially in elderly women[1]. Furthermore, a high prevalence of iron deficiency with or without anemia has been reported among patients with restless legs syndrome[2].

Etiology

The etiology of IDA can be broadly categorized into two: increased iron loss/use (acute/chronic blood loss, menses, blood donation, rapid growth during childhood, pregnancy) and decreased iron uptake (inadequate diet, malabsorption due to GI disease or surgery). Common etiologies of IDA stemming from GI diseases are shown in Table 1. Any hemorrhagic lesion within the GI tract may result in blood loss and iron deficiency. IDA is often the first sign of an occult GI malignancy, celiac disease, and gastritis[3,4].

Diagnosis

Although patients with IDA generally present with microcytic, hypochromic anemia, early IDA has normocytic, normochromic indices. Reticulocyte count (corresponding to red cell production) and ferritin level (corresponding to stored iron amount) are typically low in uncomplicated IDA. Serum iron levels are not helpful by themselves because they vary with time of the day[5] and due to various systemic insults. Serum ferritin concentration is an excellent indicator of body iron stores, however, ferritin is an acute phase reactant and its level may be elevated in chronic inflammatory states. Virtually all patients with serum ferritin concentrations less than 15 ng/mL are iron deficient, with a sensitivity and specificity of 59% and 99%, respectively[6]. A cutoff limit of 30 ng/mL may increase its sensitivity to 92%[7]. Transferrin iron binding capacity (ratio of iron-unbound transferrin to total transferrin) is a helpful marker in the diagnosis of IDA and is generally lower than 16% in patients with IDA. Several conditions may complicate the diagnosis of IDA such as infections, malignancies, chronic renal failure, and inflammatory conditions by falsely elevating ferritin levels. Newer assays such as soluble transferrin receptor level and reticulocyte hemoglobin (Hb) content may be helpful, although lack of reliable standards prevented these assays from becoming a routinely available test in clinical practice[8]. Although examination of the bone marrow is considered to be the best method for evaluation of body iron status, it is invasive, expensive, and operator-dependent. In addition, recent papers questioned its accuracy[9].

TREATMENT

Keeping the diverse etiology of IDA in mind, clinicians should not consider it as a simple ailment or deficiency but a sign of a potentially life-threatening disease. Therefore, the mainstay of IDA management should be identification and correction of the underlying pathology. The search for etiology should start with history, primarily directed to assess blood loss from the GI tract and due to obstetrical causes. Potentially curable GI malignancies can be found more commonly using colonoscopy than upper endoscopy[10]. However, the overall prevalence of upper GI lesions is higher than that of lower GI lesions in patients with IDA[11,12]. The initial diagnostic test (colonoscopy *vs* upper endoscopy) can be chosen based on findings in the history.

Red blood cell transfusions are required in hemodynamically unstable patients primarily due to acute GI bleeding. On the other hand, transfusions should be avoided in chronically anemic, hemodynamically stable patients without cardiac or pulmonary comorbidities unless the Hb level is < 7 g/dL[13] due to the potentially life-threatening side effects of transfusions as well as their cost. In case of urgency, anemia can be corrected rapidly with parenteral iron therapy in patients with uncomplicated IDA.

Furthermore, iron studies should be performed after GI bleeding resolves even in non-anemic patients since frequent bleeding may deplete body iron stores without causing explicit anemia. Iron is essential for all cells in the body; and iron replacement in non-anemic but iron deficient patients may improve quality of life[14] and cognitive function[15]; may also delay/prevent the development of IDA in patients with frequent episodes of GI bleeding such as those with IBD and angiodysplasias. Clinicians should take side effects of oral iron therapy and the frequency of patients' bleeding episodes into consideration when deciding on iron supplementation in non-anemic iron deficient patients.

Oral iron replacement

Iron stores can be replenished through oral and parenteral therapy. In asymptomatic and mildly symptomatic patients with IDA, oral iron replacement therapy has been the mainstay therapy. Various iron salts have been used, ferrous sulfate being the most common. Use of oral iron is primarily limited by its GI side effects that are mediated by non-absorbed iron. Although newer preparations were claimed to have less side effects, ferrous sulfate is still the most commonly used oral iron preparation. Accordingly, no difference in efficacy and side effect profile were found between ferrous sulfate, ferrous gluconate, and ferrous fumarate in a randomized, double blind study[16]. However, controlled-release iron preparations and polysaccharide-iron complexes were found to have fewer GI side effects than ferrous sulfate in a few randomized trials[17,18].

Ferrous sulfate is 20% elemental iron so that a 325 mg tablet contains 65 mg iron. Although conventional wisdom

Table 1 Causes of iron deficiency anemia stemming from the GI tract

Decreased iron uptake	Increased iron loss (bleeding into GI tract)
Celiac sprue	Esophagus (esophagitis and cancer)
Giardiasis	Stomach (ulcer, gastritis, cancer, vascular lesions)
Achlorhydria (due to atrophic gastritis, *H. pylori* infection…)	Small bowel (vascular lesions)
Gastrojejunostomy and other surgical techniques bypassing the duodenum where iron absorption is maximal	Large bowel (cancer, angioectasias, adenoma, colitis)
Short bowel syndrome	

GI: Gastrointestinal; *H. pylori*: *Helicobacter pylori*.

Table 2 Pros and cons of parenteral iron over oral iron therapy

Pros
Assured repletion of iron stores regardless of factors affecting iron absorption
Rapid reversal of iron deficiency
Certain or at least assessable adherence to therapy
Infrequent side effects
One-time administration (FeCarb and LMWID)
Cons
Rare lethal drug-related adverse events
Expensive
Requires facilities/staff for administration
Simply taking tablets may be more convenient for some patients

FeCarb: Ferric carboxymaltose; LMWID: Low-molecular weight iron dextran.

dictates administration of 200 mg elemental iron daily for correction of IDA, there is no rationale for using such a high dose of oral iron. Iron absorption from the GI tract is highly efficient but saturable[19]. Accordingly, Rimon *et al*[20] demonstrated that oral iron preparations at doses as low as 15 mg/d could be used to correct iron deficiency.

To avoid GI side effects and consequent non-compliance, oral iron should be started at a low dose once daily after meals then the dose can be increased at the clinician's discretion. If well tolerated, patients should attempt to take iron preparations on an empty stomach to increase iron absorption. Within 7-14 d of therapy, an increase in reticulocyte count would be expected and within 2 mo Hb level should return to normal. Oral iron replacement should be continued to replenish iron stores, usually for an additional 4 to 6 mo after Hb normalization.

Parenteral iron replacement

The parenteral route should be used for iron replacement in patients who cannot be treated adequately with oral iron supplements due to severe GI side effects, inadequate absorption, and anemia that requires urgent correction. Many clinicians have been reluctant to use parenteral iron formulations due to the infrequent, random, but rarely lethal hypersensitive reactions to high molecular weight iron dextran (HMWID) infusions. However, the incidence of severe adverse effects is lower with low molecular weight iron dextran (LMWID) and newer parenteral iron complexes. Rates of life threatening adverse drug events were reported as 0.6, 0.9, 3.3, and 11.3 per million doses of iron sucrose, iron gluconate, LMWID, and HMWID, respectively[21]. The pros and cons of parenteral over oral iron replacement are listed in Table 2.

All parenteral iron complexes consist of a ferric iron core and a stabilizing carbohydrate shell. Table 3 demonstrates the selected characteristics, administration guidelines, and side effect profiles of four parenteral iron complexes[22,23]. Following parenteral administration, the iron-carbohydrate complex is separated by the reticuloendothelial system and iron is gradually released into the circulation combining with transferrin for transport to the liver, spleen, and bone marrow. Parenteral iron is administered at a dose to restore the total iron deficit (TID) in the body. The TID is traditionally calculated by Ganzoni's formula[24]: (2.4 × body weight (kg) × [target Hb (g/dL) - observed Hb (g/dL)] + 500 mg (iron depot). However, this formula may underestimate the iron depot in males which is estimated to be 700-900 mg[25].

Iron gluconate and iron sucrose are weaker complexes than iron dextran and ferric carboxymaltose (FeCarb). Therefore, iron is released into circulation quicker and becomes readily available for erythropoiesis[26]. While early release of iron may cause a quicker response in Hb levels, it increases the risk of acute adverse events because non-bound labile iron may cause transient capillary leak syndrome leading to nausea, hypotension, tachycardia, dyspnea, and edema mimicking anaphylaxis[27]. Hence, iron gluconate and iron sucrose have lower maximal recommended single doses.

Due to the higher incidence of anaphylactic reactions associated with iron dextran, a test dose of 25 mg as slow intravenous (IV) push is required before LMWID administration. On the other hand, LMWID can be administered at higher doses enabling physicians to administer the total iron dose at one infusion. Furthermore, LMWID is less expensive compared to the other parenteral iron complexes. Iron dextran can also be administered intramuscularly, however, this is not recommended due to the incomplete absorption from the injection site and pain on injection.

FeCarb is a novel parenteral iron complex with a favorable side effect profile that can be applied at single doses up to 1000 mg per week at a high infusion speed (1000 mg IV over 15 min)[28]. However, the safety information on FeCarb is not mature and various phase Ⅲ trials are underway evaluating its efficacy and safety. As of December 2009, FeCarb has not been approved by the USA Food and Drug Administration.

Table 3 Selected characteristics and dosage guidelines of parenteral iron complexes

	LMWID	Iron gluconate	Iron sucrose	FeCarb
Concentration	50 mg/mL (2 mL vial)	12.5 mg/mL (5 mL ampule)	20 mg/mL (5 mL vial)	50 mg/mL (2 mL vial)
IV injection dose	100 mg over 2-5 min	125 mg over 10 min	100 mg over 5 min	100 mg over 2 min
Direct iron donation to transferrin	1-2	5-6	4-5	1-2
Test dose required	Yes, 25 mg slow IV push	No	No	No
Maximal single dose for IV infusion	Not limited	125	500	1000
Total dose infusion	Yes, in NS over 1-6 h	No	No	No
Pregnancy category	C	B	B	N/A
Life threatening ADEs (per 10^6 doses)	3.3	0.9	0.6	N/A

LMWID: Low molecular weight iron dextran; IV: Intravenous; ADE: Adverse drug event; NS: Normal saline; N/A: Not available.

Table 4 Clinical studies comparing parenteral and oral iron therapy

Author	Intervention	Study method	*n*	Efficacy	*P*
Schröder *et al*[33]	Elemental iron 100-200 mg/d × 6 wk	Randomized	24	[1]RR: 53%	0.85
	Iron sucrose 7 mg/kg × one dose then 200 mg once-twice/wk × 5 wk		22	[1]RR: 55%	
Erichsen *et al*[34]	Elemental iron 120 mg/d × 14 d	Crossover trial with a washout period of > 6 wk	17	Mean increase in Hb: 0.2	< 0.05
	Iron sucrose 200 mg on days 1, 5, 10		17	Mean increase in Hb: 0.7	
Kulnigg *et al*[28]	Elemental iron 200 mg/d × 12 wk	2:1 (FeCarb:oral) randomization	136	Median increase in Hb: 2.8	NS
	FeCarb 1000 mg (max) weekly (× 1-3 wk)		60	Median increase in Hb: 3.7	
Gisbert *et al*[35]	Elemental iron 106 mg/d × 3-6 mo	Hb ≥ 10.0 g/dL	78	[2]RR: 89%	NS
	Iron sucrose 200 mg twice/wk × 3-6 mo	Hb < 10.0 g/dL	22	[2]RR: 77%	
Lindgren *et al*[36]	Elemental iron 200 mg/d × 20 wk	Randomized	46	[1]RR: 47%	0.07
	Iron sucrose 200 mg weekly until calculated dose reached		45	[1]RR: 66%	

[1]Increase in Hb ≥ 2.0 g/dL; [2]Complete normalization of Hb. RR: Response rate.

IRON DEFICIENCY IN INFLAMMATORY BOWEL DISEASE

IDA in IBD requires further attention because of its complex etiology and the current debate on its treatment. Anemia is thought to be the most common extraintestinal manifestation of IBD with a prevalence of 6%-74% in different study populations (higher in hospitalized patients)[29]. Anemia in IBD is multifactorial in origin and is frequently due to the combination of iron deficiency (primary cause) and anemia of chronic disease (ACD). In some cases, anemia may be drug induced (mesalazine, sulfasalazine, azathioprine, mercaptopurine) or due to folate/vitamin B12 deficiency. Iron deficiency in IBD is primarily caused by chronic blood loss through the GI tract, however, decreased iron absorption may also play a role in patients with Crohn's disease affecting the proximal small bowel. The prevalence of iron deficiency in IBD ranges from 36% to 90% depending on the definition of iron deficiency and cohort selection[30-32].

The frequent mixture of IDA and ACD in patients with IBD creates a diagnostic dilemma. Chronic inflammation increases serum ferritin and decreases serum transferrin level. As a result, the sensitivities of ferritin and iron saturation for IDA are decreased in patients with IBD. In addition to serum ferritin level and iron saturation, clinicians may use novel tests such as soluble transferrin level or a therapeutic trial of iron replacement therapy in anemic IBD patients.

The current debate on treatment of IDA in patients with IBD centers on the choice of the iron replacement route. The advantages of parenteral over oral iron replacement are thought to be:

Parenteral therapy may be more efficacious than oral supplementation because of low iron absorption from the GI tract in patients with IBD and lower compliance to the oral therapy. However, currently available data are not sufficient to reach such a conclusion. A few studies comparing the efficacy of oral and parenteral iron therapy have been published with conflicting results[28,33-36] (Table 4). Although Lindgren *et al*[36] recently concluded that IV iron therapy was more efficacious in the treatment of IDA in IBD after an intent-to-treat analysis, this study was confounded by a high discontinuation rate in the oral therapy arm (11 of 46 patients).

Persistent blood loss may exceed the capacity of intestinal absorption of iron in some patients[37]. The maximal iron absorption from intestines depends primarily on the level of body iron store and iron intake. Iron-deficient patients receiving 100 mg elemental iron/d can absorb at most 25-37.5 mg iron/d[38,39]. Since whole blood contains 0.5 mg elemental iron/mL[40], oral iron therapy may replace the iron lost in 50-75 mL of blood in the best circumstances. Therefore, clinicians should not expect oral iron therapy to correct IDA and replenish body iron stores in patients losing more than 20-30 mL blood daily. Consequently, parenteral therapy may be more useful in patients with moderate-severe active IBD.

Parenteral therapy causes an earlier increase in Hb levels[28,34]. For that reason, it is more valuable in situations where rapid correction of anemia is required such as in hemodynamically stable patients with moderately severe symptoms of anemia or those requiring an elective procedure within a few weeks.

Oral iron therapy has a high incidence of GI side effects such as epigastric pain and diarrhea that lead to discontinuation of therapy in up to 21% of patients[29]. Additionally, it is extremely difficult to differentiate these side effects from IBD exacerbation. Blackened stools by non-absorbed iron may also mimic melena and lead to unnecessary tests. On the other hand, lower doses of iron may be better tolerated and as efficacious as higher doses. Gisbert *et al*[35] recently reported a discontinuation rate of 5% and normalization of Hb concentration in 89% of IBD patients treated with 106 mg of oral elemental iron daily.

In addition to its GI side effects, oral iron may worsen IBD as a result of non-absorbed iron-mediated toxic reactive oxygen species. Accordingly, oral iron therapy was demonstrated to worsen disease activity in several rodent models at doses 100- to 1000-fold higher than the therapeutic iron dose[41-44]. In the only animal study in which the clinically therapeutic dose in humans was used, oral iron was found to increase histologic colitis scores in DSS-induced colitis in rats[45]. Furthermore, oral iron therapy was found to worsen IBD activity indices in one clinical study[34] while it improved or had no effect in other studies[35,36,46]. Irrespectively, disease activity questionnaires in IBD patients being treated with oral iron may not reflect the true disease activity but may be confounded with the GI side effects of iron.

On the other hand, oral iron therapy is less expensive, does not require additional infusion staff and infusion facilities, does not have life-threatening side effects, and may be more convenient for some patients because of logistics. We believe parenteral iron therapy is still advantageous over oral therapy in patients with IBD due to a quicker response, efficacy in severe cases, and the GI side effects of oral therapy.

Another pressing issue on iron replacement is the duration of therapy. Kulnigg *et al*[47] recently reported that anemia recurred within 10 mo of therapy cessation in 50% of anemic IBD patients whose Hb levels had normalized after IV iron sucrose and erythropoietin therapy, indicating a need for continuous iron maintenance therapy. In addition, patients with a ferritin level > 400 mg/dL at the end of replacement therapy had a longer period until they become iron deficient. Therefore, after replenishing TID, clinicians may opt to continue iron therapy at a less frequent maintenance dose or to switch to low-dose oral therapy.

Today, the widely recommended indications for use of IV iron in IBD patients with IDA are: severe anemia (defined as Hb level < 10 g/dL), need of quick recovery, intolerance to oral iron, and failure of oral iron. We agree with all of these indications with the exception of setting a definite Hb threshold. Considering the multifactorial etiology of anemia in IBD and the widely different tolerances of people to anemia, we believe that the choice of the iron administration route should primarily depend on the severity and acuity of patients' signs and symptoms. Concentrating only on the Hb level and overlooking the factors that may affect patients' tolerance to anemia such as age, cardiac and pulmonary comorbidities may blur the bigger picture and lead to over- or under-treatment. We believe symptoms such as dyspnea on mild exertion, tachycardia, severe fatigue, and ongoing gross blood loss from the GI tract should urge the clinicians to recommend first-line parenteral iron therapy to their patients with IBD and IDA.

CONCLUSION

GI diseases are one of the most common etiologies of IDA. Iron replacement should be initiated in patients with IDA and iron deficient non-anemic patients with recurrent episodes of GI bleeding. Novel parenteral iron preparations are safer than high molecular weight iron dextran and are more useful in patients with IBD primarily due to GI side effects of oral iron therapy. Finally, regardless of the route, iron therapy needs to be continued until iron stores are completely replenished. The need for maintenance therapy should be assessed in prospective trials.

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TOPIC HIGHLIGHT

Yusuf Bayraktar, Professor, *Series Editor*

Molecular basis and management of gastrointestinal stromal tumors

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Abstract

Molecularly targeted agents have dramatically impacted the management of several cancers. Targeting KIT has led to a new treatment paradigm in gastrointestinal stromal tumors (GISTs). KIT is a cell surface receptor with tyrosine kinases that, upon binding of its ligand, stem cell factor, activates various signaling pathways. Imatinib and sunitinib, both tyrosine kinase inhibitors directed to KIT, were approved for first- and second-line treatment of metastatic and unresectable GISTs. In this article, we will review the molecular pathogenesis of GISTs followed by a discussion of imatinib and sunitinib's role in the treatment of GISTs. Finally, we will introduce novel therapeutic options for imatinib- and sunitinib-resistant GISTs.

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Key words: Gastrointestinal stromal tumor; KIT; Imatinib; Sunitinib; Nilotinib

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INTRODUCTION

The gastrointestinal stromal tumor (GIST) is the most common (80%) mesenchymal neoplasm of the gastrointestinal tract[1] and represents about 5% of all sarcomas[2]. The annual incidence of GISTs is estimated to be 14.5 per million and prevalence 129 per million[3], with as many as 6000 new cases per year in the United States[4].

GISTs represent a morphological and biological gamut from incidentally discovered, < 1 cm nodules of benign appearance to large sarcomas, although all GISTs are currently accepted as potentially malignant. GISTs most commonly involve the stomach (50%) and small bowel (25%) (Figure 1); however, they may occur anywhere in the GI tract including the mesentery and omentum[5,6]. Approximately half of the patients present with metastatic disease and nearly two-thirds of those have liver metastases. Extra-abdominal and lymph node metastases at presentation are rare, obviating the need for lymph node dissection unless they are directly involved[2].

Until the 1980s, stromal tumors arising from gastrointestinal tract were classified as various entities such as leiomyosarcoma, leiomyoblastoma and bizarre leiomyoma. With the advent of immunohistochemistry, it was realized

that these tumors lacked immunophenotypic characteristics of smooth muscle. Mazur and Clark showed that a proportion of these tumors stained positively for S-100 and suggested the myenteric nervous system as the cells of origin, introducing the more generic term, "stromal tumor"[7]. In 1998, Hirota and colleagues demonstrated that almost all GISTs expressed KIT and had activating c-kit mutations. They also showed that interstitial cells of Cajal (ICC) were positive for both KIT and CD34 suggesting ICC as the cells of origin of GISTs[8].

KIT

KIT encoded by the c-*Kit* gene (mapped to chromosome 4q12) belongs to the type Ⅲ receptor tyrosine kinase family and is structurally similar to platelet-derived growth factor receptors (PDGFRs), colony-stimulating factor-1 receptor, and fms-like tyrosine kinase-3 (FLT-3)[9,10]. KIT consists of an extracellular (EC) domain with 5 immunoglobulin-like loops, a transmembrane region, and a cytoplasmic domain with juxtamembrane (JM) region and a split tyrosine kinase (TK) domain. The latter is divided into an adenosine triphosphate (ATP) binding region (TK1) and a phosphotransferase region (TK2) by a hydrophilic kinase insert (KI) (Figure 2).

Normally, KIT is activated by stem cell factor. Ligand binding to the EC domain results in the dimerization of receptors and phosphorylation of tyrosine in the cytoplasmic TK domains. This leads to a phosphorylation cascade and activation of signal transduction pathways including Ras/MAP kinase, Rac/Rho-JNK, PI3K/AKT and SFK/STAT signaling networks[11]. Signaling by KIT plays an important role in erythropoesis, lymphopoesis, mast cell development and function, megakaryopoesis, gametogenesis and melanogenesis.

KIT is expressed in more than 95% of GISTs, including tumors with wild-type KIT and most PDGFR-A mutant GISTs. However, in the latter group, KIT expression may be weaker. KIT mutation in GISTs does not cause KIT expression, but modifies KIT function[12]. About 65%-85% of GISTs have KIT mutations[13-15]. Based on the location, these mutations could be divided into 2 categories: mutations of the receptor regulatory domain (EC and JM) and mutations of the enzymatic domain (TK1 and TK2)[16]. Most involve the JM domain (exon 11) and consist mostly of deletions or point mutations. Mutations in the JM domain affect KIT's autoregulatory function and promote spontaneous kinase activation[17]. Exon 9 (EC domain) mutations are the second most common mutations followed by exon 13 (TK1 domain) and exon 17 (TK2 domain) mutations.

In approximately 10% of GISTs, no c-kit mutations are found even when the whole coding region is examined. In 2003, Heinrich *et al*[18] and Hirota *et al*[19] found gain-of-function mutations of the *PDGFR-A* gene in about half of the GISTs lacking c-kit mutations. A majority of PDGFR-A mutations affect the TK2 domain (exon 18). These mutations change the activation loop, which regulates the ATP-binding pocket and leads to kinase activation[6]. Activated PDGFR-A activates the same signaling pathways that KIT activates, leading to GIST.



Figure 1 Endoscopic appearance of a jejunal gastrointestinal stromal tumor (GIST).

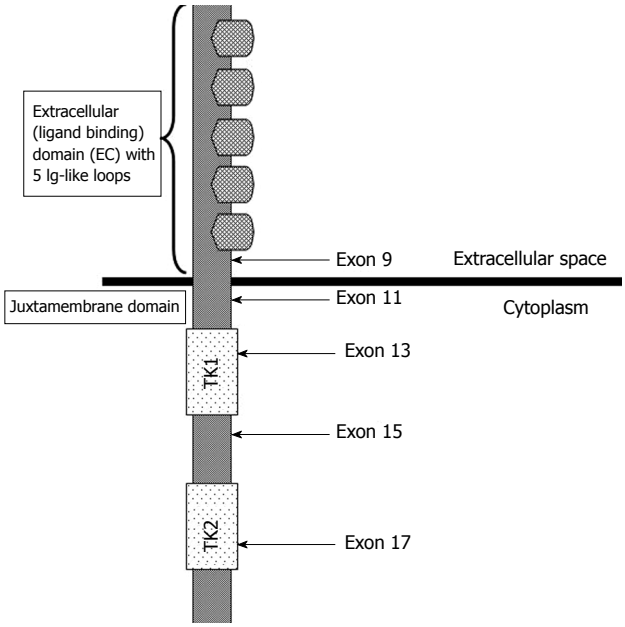


Figure 2 KIT structure and localization of common KIT mutations. Ig: Immunoglobulin.

IMATINIB

Imatinib mesylate (henceforth referred to as imatinib) is a small-molecule TK inhibitor. It works by binding to ATP binding sites of KIT, PDGFR-A, and bcr-abl fusion product, consequently inhibiting their activity[20].

Imatinib is rapidly absorbed in the GI tract, reaching maximum plasma concentrations 2-4 h after oral intake. Absolute bioavailability of imatinib is 98% and food does not affect its absorption[21]. Imatinib is metabolized mainly by the cytochrome P450 (CYP) 3A4 enzymes in the liver, with minor contributions from other CYP enzymes and is eliminated mainly via the feces, mostly in the form of metabolites[22]. As a result of intrinsic variability of CYP enzyme activity, there is a high inter-patient variability for imatinib exposure. Accordingly, imatinib trough levels at

day 29 were recently reported to be associated with clinical outcome[23]. Concomitant administration of CYP3A4 inhibitors (e.g. ketoconazole) may increase and CYP3A4 inducers (e.g. rifampin) may decrease imatinib systemic exposure. Conversely, imatinib may inhibit the metabolism of CYP3A4 substrates (e.g. simvastatin), thereby increasing their exposure. Imatinib may also increase systemic exposure to paracetamol by inhibiting its O-glucuronidation[21,24].

In May 2001, the US Food and Drug Administration (FDA) approved imatinib for chronic myelogenous leukemia (CML) based on excellent responses as first- and second-line treatments[25]. Following demonstration of its activity against KIT and successful treatment of a patient with a GIST[26], imatinib was evaluated in an open label, randomized, multicenter trial. Of 147 patients with unresectable or metastatic GISTs who were randomly assigned to receive either 400 or 600 mg imatinib daily, 79 (54%) had a partial response and 41 (28%) had stable disease. None of the patients had a complete response. There were no significant differences in the rate or duration of responses between the 2 dose levels of imatinib that were tested. The estimated 1-year overall survival rate was 88% for all patients[27]. As a result of this study, the FDA approved the use of imatinib in advanced GISTs in 2002. Recently, a long-term follow-up analysis of the same trial demonstrated that median duration of response was 27 mo and median overall survival was 58 mo[28].

In the same phase Ⅱ trial, the 18F-fluoro-2-deoxy-D-glucose uptake in the tumors was found to be decreased markedly from baseline as early as 24 h after a single dose of imatinib. The median time to an objective response was 13 wk[27]. Accordingly, in the Euro-Australasian phase Ⅲ trial, the median time to best response was 15 wk, with most responses occurring within the first 9 mo. However, best responses were occasionally observed after as long as 2 years of treatment suggesting that responses to imatinib can be seen late in the treatment[29].

Blay *et al*[30] investigated whether imatinib could be stopped after 1 year in patients who responded to imatinib and concluded that treatment with imatinib should continue until disease progression, patient intolerance or refusal. In this study, 58 patients with advanced GISTs were randomized to continuous and intermittent treatment arms. Twenty-six (81%) of the 32 patients in the intermittent arm had progressed and were restarted on imatinib. Of these 26 patients, 24 had a response or stable disease after imatinib was restarted. At the time of the final analysis, there was no difference in overall survival between continuous and intermittent treatment arms[30].

Adjuvant and neoadjuvant therapy with imatinib

Imatinib showed promising results in adjuvant therapy of GISTs. However, there is an ongoing debate on the duration, dose, and the selection of patients for adjuvant imatinib therapy. It is assumed that GIST patients with a relatively high risk of recurrence would benefit the most from adjuvant treatment. Several models were constructed to estimate the recurrence risk of resected GISTs.

Table 1 Risk stratification of primary GISTs

Tumor feature		Risk of tumor progression	
Mitotic index	Size (cm)	Stomach	Small bowel[1]
< 5/50 HPF	≤ 2	Very low	Very low
	> 2 ≤ 5	Very low	Low
	> 5 ≤ 10	Low	Moderate
	> 10	Moderate	High
≥ 5/50 HPF	≤ 2	Very low	Moderate
	> 2 ≤ 5	Moderate	High
	> 5 ≤ 10	High	High
	> 10	High	High

Modified from Hornick *et al*[33] Copyrighted permission from Elsevier Inc. [1]GISTs arising from other sites should probably be stratified as small bowel tumors. GISTs: Gastrointestinal stromal tumors; HPF: High power fields.

Parameters commonly used were tumor size and mitotic rate[4,31]. Based on the reports of a higher recurrence risk of small bowel GISTs compared to gastric ones[32], a new risk stratification model incorporating tumor location was proposed[12,33] (Table 1). In addition to estimation of the recurrence risk, in the near future, mutation analyses from tumor specimens may help practitioners to select different agents according to different genotypes, customize both the duration and the dose of imatinib or other effective agents in the adjuvant setting.

After a phase Ⅱ trial (ACOSOG Z9000) demonstrated high tolerability and promising efficacy in high risk (tumor size > 10 cm, tumor rupture, or multifocal disease) GIST patients[34,35], a phase Ⅲ trial (ACOSOG Z9001) was initiated to compare 400 mg imatinib daily for 1 year with placebo in patients with KIT-expressing GISTs measuring at least 3 cm that were grossly resected. Accrual to the trial was halted early because of the better relapse-free survival (RFS) in the treatment arm in the interim analysis, and these results were presented at the 2007 ASCO meeting leading the FDA to approve imatinib for adjuvant treatment of GISTs in the USA[36]. Final results showed a 1-year RFS of 97% and 83% in imatinib and placebo arms, respectively, with a hazard ratio (HR) of 0.35 (95% confidence interval, 0.22-0.53)[37]. However, the risk reduction in patients with a tumor diameter between 3 and 6 cm was nearly zero. Secondly, there was no difference in overall survival between the 2 groups. It remains unclear whether imatinib prevents GIST recurrence or merely delays it. While 2 phase Ⅱ trials from Korea and China confirmed the improved RFS in intermediate- and high-risk GIST patients treated with imatinib in an adjuvant setting[38,39], 2 other phase Ⅲ trials are still ongoing: EORTC 62024 comparing imatinib for 2 years *vs* placebo, and SSG XVⅢ/AIO trial comparing imatinib for 3 years *vs* 1 year. Long-term follow-up results will be needed to demonstrate the adjuvant treatment effect on overall survival, the subsets of patients that would benefit, and the optimal duration of the treatment. Table 2 demonstrates the adjuvant/neoadjuvant trials registered by the National Cancer Institute (USA).

On the neoadjuvant side, early results from the phase Ⅱ RTOG 0132 trial established the feasibility of the ap-

Table 2 Adjuvant and neoadjuvant trials of imatinib in patients with GISTs

Trial	Accrual	Eligibility	Therapy (*n*)	End points
Phase Ⅱ study of adjuvant imatinib mesylate in patients with completely resected high-risk primary GIST (ACOSOG-Z9000)	Closed	Diameter > 10 cm or tumor rupture or multifocal	Imatinib 400 mg daily for 1 year (107)	2-year OS: 97%, 2-year RFS: 73%[33]
Phase Ⅲ randomized study of adjuvant imatinib mesylate in patients with resected primary GIST (ACOSOG-Z9001)	Closed	Diameter > 3 cm	Imatinib 400 mg daily for 1 year (359) Placebo (354)	1-year RFS: 98%[36] 1-year RFS: 83%
EORTC soft tissue and bone sarcoma group (EORTC-62024) randomized phase Ⅲ trial	Closed	Diameter > 5 cm or mitotic rate > 5/50 HPF	Imatinib 400 mg daily for 2 years Observation (Total projected 750)	Primary: OS Secondary: RFS and safety
Scandinavian sarcoma group trial SSGXVⅢ	Closed	Diameter > 10 cm or mitotic rate > 10/50 HPF or > 5 cm and > 5/50 HPF or tumor rupture	Imatinib 400 mg daily for 36 mo Imatinib 400 mg daily for 12 mo (Total projected 280)	Primary: RFS Secondary: OS, safety
Phase Ⅱ study of neoadjuvant and adjuvant imatinib mesylate in patients with primary or recurrent potentially resectable malignant GIST (RTOG-S0132)	Closed	Locally advanced or metastatic/recurrent	Imatinib 600 mg daily for 6-8 wk followed by debulking/resection (52)	2-year PFS: 80%, objective response rate: 6%, R0 resection in 65%[39]
Five year adjuvant imatinib mesylate in GIST (Phase Ⅱ)	Open	Diameter > 2 cm and mitotic rate > 5/50 HPF or non-gastric GIST > 5 cm	Imatinib 400 mg daily for 5 years (Projected 133)	Primary: Time to recurrence Secondary: Safety
Phase Ⅱ study of neoadjuvant imatinib mesylate in patients with locally advanced gastrointestinal stromal tumor (Germany/Austria)	Open	Locally advanced, KIT expressing, histologically confirmed GIST	Imatinib 400 mg daily/*BID* (Projected 40)	Primary: ORR Secondary: R0-resectability and organ-preserving resectability

OS: Overall survival; RFS: Relapse-free survival; PFS: Progression-free survival.

proach in locally advanced and metastatic GIST patients[40]. Fiore *et al*[41] recently reported that all of the 15 patients who received preoperative imatinib benefited from neoadjuvant treatment: one patient had a complete response; 3 patients who were initially considered to have unresectable disease underwent complete resection; 7 patients with initial indication for extensive surgery were more conservatively operated on; 4 patients initially deemed at high perioperative risk underwent safe surgery[41]. For patients that do not have access to the ongoing neoadjuvant trials (Table 2), we consider preoperative imatinib as an option for those with unresectable GISTs or in whom surgical morbidity would be improved by reducing the size of the tumor preoperatively. Positron emission tomography (PET) scans within 2-4 wk of therapy could be a predictive test separating responding from resistant patients. Non-responding patients, if surgically resectable, would be taken to surgery. Patients that are not surgical candidates could be considered for second-line therapy.

Toxicity

Almost all patients in clinical trials with imatinib experienced adverse events; however, most of the events were mild or mild to moderate. Patients treated with 800 mg/d imatinib had more grade 3 or above side effects compared to those treated with 400 mg/d in both phase Ⅲ trials comparing 2 different doses. Most common grade 3-4 side effects in these 2 phase Ⅲ trials were anemia, granulocytopenia, fatigue, fluid retention, muscle pain, and gastrointestinal symptoms[29,42]. In the Euro-Australasian trial, dose reductions and treatment interruptions, mostly because of toxic effects, were found to be more likely to occur in patients who received imatinib 400 mg twice daily. Additionally, side effects were mostly recorded during the first 8 wk of treatment[43].

Fluid retention due to imatinib is generally mild, most frequently affects the periorbital region, and can be seen in 70%-80% of patients treated with imatinib. It responds well to intermittent low dose thiazide diuretics. On the other hand, muscle cramps can be seen in up to 40% of patients and can be very troublesome as a result of their long-term course. There are anecdotal reports of patients responding to magnesium/calcium supplements[44], quinine[45], and chlordiazepoxide[46]. Hypophosphatemia can also be seen with imatinib use, and routine monitoring of phosphate levels has been suggested[47]. In 2006, 10 individuals were reported to develop severe heart failure after receiving imatinib[48]. However, whether imatinib causes cardiac toxicity in GIST patients is still unclear. Multiple randomized studies failed to show excess cardiotoxicity in patients treated with imatinib postoperatively[37,49]. Tumor hemorrhage is a life-threatening toxicity for which patients with large, bulky tumors have a higher risk. Therefore, hemoglobin levels should be monitored upon starting GIST patients on imatinib.

Imatinib resistance

The advent of imatinib had completely changed the management and survival of patients with GISTs. However, imatinib resistance, both primary and secondary, is still a

Table 3 Prevalence of c-kit and PDGFR-A mutations and clinical responses in advanced GISTs to imatinib and sunitinib correlated with mutational status

Genotype	Prevalence[59-61]	Clinical benefit from imatinib[15,58,59]	Clinical benefit from sunitinib[58]1
KIT exon 9 mutation (%)	5-14	74-81	60
KIT exon 11 mutation (%)	57-69	83-93	35
KIT exon 13 mutation (%)	< 5	60-100 (few cases)	65
KIT exon 17 mutation (%)	< 5	75-80 (few cases)	
PDGFR-A mutations (%)	3-8	40-66	0 (few cases)
Both wild-type (%)	5-10	33-73	55

Clinical benefit is defined as complete, partial response or stable disease. [1]In imatinib-resistant GIST.

significant problem. In the Euro-Australasian phase Ⅲ trial, 12% of patients exhibited initial resistance to imatinib[29]. Furthermore, more than 40% of patients who were initially responsive to imatinib developed late resistance after a median follow-up of 25 mo.

Several different mechanisms of imatinib resistance were identified in the literature: (1) KIT or PDGFRA mutations resulting in intrinsic target resistance; (2) genomic amplification leading to target overexpression; and (3) alternate oncoprotein activation supplanting target expression[24,50,51]. During imatinib treatment, resistance often develops as a result of secondary mutations, primarily in the kinase domains of KIT or PDGFR-A[52-54]. One of the frequent secondary kinase domain mutations, V654A mutation, is intrinsically imatinib-resistant[55].

Depending on the mechanism of the resistance, a higher dose of imatinib may be considered in patients with GISTs resistant to 400 mg/d imatinib. The effect of increasing the dose of imatinib to 800 mg daily in advanced GIST patients was assessed in 2 phase Ⅲ trials. In the Euro-Australasian trial of 946 patients, Verweji *et al*[29] reported that although there was no difference in response rates between daily and twice daily 400 mg dosing, progression-free survival was significantly better in patients who received the higher dose. On the other hand, the results of the phase Ⅲ trial in North America did not reveal any difference between daily and twice daily 400 mg dosing in response rates, progression-free and overall survival. However, 31% of patients who progressed on 400 mg daily dosing and crossed over to 400 mg twice daily dosing had a partial response or stable disease suggesting that in a subset of patients increasing the imatinib dose may overcome the resistance to imatinib[42].

In the North American phase Ⅲ trial, patients with exon 11 mutations had a better response rate, time to progression and overall survival compared to those with exon 9 mutations and the wild-type *c-kit* gene. Although the survival rates of exon 9, exon 11 or wild-type GISTs were not affected by imatinib dose, the response rate for patients with an exon 9 mutation was significantly higher in those who were treated with a higher dose of imatinib (67% *vs* 17%, $P = 0.02$)[56]. Furthermore, in a meta-analysis of 2 phase Ⅲ trials, 800 mg/d imatinib was found to be associated with a significantly better progression-free survival in patients with the exon 9 mutation (median progression-free survival: 1.6 year *vs* 0.5 year; HR, 0.58, $P = 0.017$)[57]. Table 3 demonstrates the prevalence and response rates of mutations in different exons of *KIT* and *PDGFR-A* genes derived from 3 different clinical trials[15,58,59], population[60] and institution based studies[61]. Lasota *et al*[62] had published a detailed review on the role of *c-kit* and *PDGFR-A* gene mutations on GIST therapy[62].

SUNITINIB

Sunitinib malate, an oral multitargeted inhibitor of KIT, PDGFRs, vascular endothelial growth factor receptors (VEGFR), FLT3 and RET receptor TKs, is FDA approved for treatment of imatinib-resistant GISTs and of advanced renal cell carcinoma. In addition to its antitumor effect through inhibition of KIT and PDGFR, sunitinib may have an antiangiogenic effect on GISTs through inhibition of VEGFR[63].

In a phase Ⅰ/Ⅱ trial, sunitinib at different dosages and schedules was administered to 97 GIST patients with imatinib resistance (96%) or intolerance (4%). In phase Ⅰ, sunitinib 50 mg/d for 4 wk followed by 2 wk off treatment was chosen for further development. Overall, 73% of patients experienced stable disease which lasted > 6 mo and 7% achieved a partial response. The median overall survival was 19 mo[64].

The pivotal, randomized, placebo-controlled, multinational phase Ⅲ trial of sunitinib in patients with unresectable, imatinib-resistant GISTs had to be unblinded early when interim data analysis showed a significantly longer median time to progression in patients who received sunitinib compared to those who received placebo (27.3 wk *vs* 6.4 wk; HR, 0.33, $P < 0.001$). Response rate was better in patients treated with sunitinib compared to those treated with placebo (6.8% *vs* 0%, $P = 0.006$). Despite the crossover after unblinding, overall survival was superior in the sunitinib arm (HR, 0.49; $P < 0.007$). This study established the role of sunitinib as a second-line therapy in patients with advanced imatinib-refractory and imatinib-intolerant GISTs[65].

Although a clinical benefit of sunitinib treatment was observed in all major mutant types, the primary response rate was significantly higher for KIT exon 9 mutants. The inhibitory effect of sunitinib was not substantially affected by KIT mutations in TK1 whereas GISTs with KIT-TK2 mutations were resistant to sunitinib treatment. Specifically, the imatinib-resistant KIT mutation V654A was highly sensitive to sunitinib[66,67].

Table 4 Novel agents being developed for GIST therapy

Agent	Molecular target	Clinical benefit in pilot studies
Kinase inhibitors		
Nilotinib	KIT, PDGFRs, bcr-abl	46%-77%[69,70]
Sorafenib	Raf, KIT, PDGFR-B, VEGFR, FLT3, RET	71%[71]
Dasatinib	Src, ABL, KIT, PDGFRs	Phase Ⅱ ongoing in advanced sarcomas and accepting patients
AZD2171	VEGFR, KIT, PDGFRs	Phase Ⅱ ongoing, not recruiting
OSI-930	VEGFR, KIT	Phase Ⅰ ongoing, not recruiting
PTK787	VEGFR, KIT, PDGFRs	67%[74]
XL820	KIT, PDGFR-B, VEGFR	Phase Ⅱ ongoing, not recruiting
AMG706	VEGFR, KIT, PDGFRs, RET	24%-27%[75,76]
mTOR and AKT inhibitors		
Perifosine	AKT	Phase II ongoing in combination with imatinib
Everolimus	mTOR	26%[73]
Temsirolimus	mTOR	Phase II ongoing, closed recruitment
Others		
IPI-504	Hsp90	78%[72], phase Ⅲ ended due to safety concerns
Flavopiridol	Transcription inhibitor	Phase Ⅰ ongoing in combination with doxorubicin

Clinical benefit is defined as complete or partial response or stable disease.

Sunitinib was generally well tolerated in patients with GISTs, with adverse events mild to moderate in severity. In the phase Ⅲ trial, the most frequent treatment-related adverse events were fatigue, diarrhea, hand-foot syndrome, rash, nausea, anorexia, and skin discoloration. Hypertension occurred in about 20% of treated patients, 5% being grade 3/4 in severity. Treatment discontinuation because of adverse events occurred in 9% and 8% of sunitinib and placebo recipients, respectively[65]. Hypothyroidism, possibly secondary to inhibition of the protein product of the RET proto-oncogene found in normal thyroid has been described in as high as 36% of sunitinib-treated GIST patients[68].

FUTURE DIRECTIONS

Currently, there is no established third-line treatment in patients with GISTs who failed to respond to both imatinib and sunitinib. Table 4 demonstrates the drugs in the pipeline for GIST therapy[69-76]. Nilotinib, a second-generation TK inhibitor engineered to inhibit KIT, PDGFR-A, and bcr-abl, is approved for the treatment of imatinib-resistant CML. In a phase I study of nilotinib in imatinib-resistant GISTs, 13 (72%) of the 18 patients who received nilotinib experienced stable disease lasting more than 6 wk. One patient achieved a partial response. Median progression-free survival was 25 wk. Nilotinib 400 mg twice daily was the dose recommended for future studies[69]. Recently, Montemurro *et al*[70] reported a retrospective analysis of 52 patients treated with nilotinib 400 mg twice daily as compassionate use in 12 European centers. All patients had failed both imatinib and sunitinib either due to resistance (96%) or intolerance (4%). One and 4 patients achieved complete and partial responses, respectively; whereas 19 had disease stabilization. The median progression-free survival was 12 wk (range 2 d-104 wk) and the median overall survival was 34 wk (range 2-135 wk). Most patients tolerated nilotinib well except 6 (12%) patients discontinued treatment due to grade 2-3 adverse effects[70].

The efficacy of sorafenib, a multi-kinase inhibitor of raf kinase, VEGFR, PDGFR, and KIT, in imatinib- and sunitinib-resistant GISTs was studied in a phase Ⅱ trial of 26 patients. Three (13%) patients achieved a partial response and 14 patients (58%) experienced stable disease. Median progression-free survival was 5.3 mo and median overall survival was 13.0 mo. The most common side effects were hand-foot syndrome (28%), hypertension (24%), and rash (20%)[71].

Heat shock proteins (Hsp) control the proper folding, function, and stabilization of various proteins. Laboratory studies have demonstrated that inhibition of Hsp90 results in selective destruction of the mutated KIT in human GIST cell lines. In a phase Ⅰ trial of IPI-504, an intravenous Hsp90 inhibitor, 78% of evaluable patients with imatinib-resistant (100%) and sunitinib-resistant (95%) GISTs experienced stable disease[72]. However, a phase Ⅲ trial of IPI-504 ended early because of safety concerns. Other strategies to inhibit growth of GISTs is to abrogate KIT mRNA expression with flavopiridol, a transcriptional inhibitor[77]; and inhibition of the AKT-mTOR pathway by mTOR inhibitors such as everolimus[73].

CONCLUSION

The discovery of near universal KIT protein expression, and activating KIT and PDGFR-A mutations advanced our understanding of GISTs significantly. Later, the finding of the high efficacy of imatinib in the treatment of GISTs changed the management and prognosis of this once very deadly malignancy. Imatinib is currently approved for the treatment of unresectable or metastatic GISTs, however, many centers around the world, including our own, use imatinib also in adjuvant and neoadjuvant settings. PET scans may be especially helpful in identifying imatinib-resistant tumors during neoadjuvant treatment. Further

studies are needed to tailor the imatinib treatment in these settings. On the other hand, imatinib should be continued until disease progression or intolerability in patients with metastatic or unresectable GISTs.

The standard therapy for metastatic GISTs resistant to imatinib is sunitinib. The efficacy of sunitinib in the first-line treatment of advanced GISTs and as adjuvant/neo-adjuvant treatment should be studied in randomized trials. Resistance to imatinib and sunitinib continues to be a challenging issue. Upcoming treatment options are second-generation kinase inhibitors and inhibition of the growth of GISTs via pathways other than KIT and PDGFR-A. We believe the combination of Hsp90 inhibitors and kinase inhibitors could yield superior results.

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REVIEW

Magnetic resonance imaging for local complications of acute pancreatitis: A pictorial review

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Abstract

Acute pancreatitis is a common disease characterized by sudden upper abdominal pain and vomiting. Alcoholism and choledocholithiasis are the most common factors for this disease. The choice of treatment for acute pancreatitis might be affected by local complications, such as local hemorrhage in or around the pancreas, and peripancreatic infection or pseudoaneurysm. Diagnostic imaging modalities for acute pancreatitis have a significant role in confirming the diagnosis of the disease, helping detect the extent of pancreatic necrosis, and for diagnosing local complications. Magnetic resonance imaging (MRI) might be indicated in acute pancreatitis for detecting and characterizing local complications of acute pancreatitis that involve necrotic, hemorrhagic, infectious, vascular, and pseudocyst disorders. The general MRI sequences for pancreatitis require the combined use of T1-weighted, T2-weighted sequences, and magnetic resonance cholangiopancreatography. For imaging of pancreatic necrosis, the combination of T1-weighted and T2-weighted findings with dynamic contrast-enhanced imaging gives a comprehensive evaluation of the extent of necrosis and full range of inflammatory extension. For imaging of infectious complications, dynamic contrast-enhanced examinations might help differentiate pancreatic cellulitis or abscesses, from pancreatic fluid collection or simple pseudocysts. For vascular abnormalities, the combination of cross-sectional pancreatic parenchyma imaging with MRA represents a single diagnostic modality for the full evaluation of peripancreatic artery and vein involvement, such as arterial pseudoaneurysms and venous thromboses. The purpose of this pictorial review is to examine the MRI appearances of various local complications of acute pancreatitis and to discuss the practical setup of MRI in local complications of acute pancreatitis.

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Key words: Pancreas; Pancreatitis; Complication; Magnetic resonance imaging; Pictorial review

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INTRODUCTION

Acute pancreatitis is a common and protean disease characterized by sudden upper abdominal pain and vomiting,

and is triggered by the leakage of activated pancreatic digestive enzymes. Alcoholism and choledocholithiasis are the most common physiological factors for acute pancreatitis[1,2]. Traditional management of acute pancreatitis tends to promote the use of conservative management. However, the choice of treatment can be influenced by local complications, such as local hemorrhage in or around the pancreas, and peripancreatic infection or pseudoaneurysm[2,3].

Increased levels of serum and/or urinary pancreatic amylase and lipase have been detected in most individuals with acute pancreatitis after the onset of symptoms. Therefore, acute pancreatitis is often diagnosed by clinical manifestations and laboratory examinations. However, diagnostic imaging modalities for acute pancreatitis have a significant role in confirming the diagnosis of the disease, helping detect the extent of pancreatic necrosis, and for diagnosing local complications[2,3].

Among a variety of imaging tools, computed tomography (CT) is considered the standard of reference for patients with acute pancreatitis; however, it has an increased radiation burden that can result from follow-up examinations[4] and displays different results for the potential aggravation of acute pancreatitis resulting from the use of iodinated contrast media[5-7]. The specific reasons for using magnetic resonance imaging (MRI) are as follows: (1) MRI is a diagnostic imaging method with no radiation hazard, which might be suitable for patients with multiple follow-up reviews; (2) MRI is a reliable method of staging the severity of acute pancreatitis, which has predictive value for the prognosis of the disease. It also has fewer contraindications than CT[8]; (3) MR cholangiopancreatography (MRCP) has the unique capability of providing noninvasive images of the pancreatic ducts and can demonstrate possible communication of a pancreatic pseudocyst with pancreatic ducts[9]; and (4) MRI in combination with MR angiography (MRA), a single diagnostic modality, is useful for assessing the signal consistency of fluid exudation or pseudocysts to identify local hemorrhage in or around the pancreas or pseudoaneurysm, which might help plan the surgery.

However, the possible drawbacks of MRI with respect to CT are cost and time consumption. Moreover, several MRI sequences require patients to hold their breath, which might be difficult for patients with severe acute pancreatitis.

MRI of pancreatitis and its complications has been reported[10]. However, to our knowledge, there is no report thoroughly assessing MRI for local complications of acute pancreatitis, including necrotic, hemorrhagic, infectious, vascular, and pseudocyst disorders.

In this pictorial essay, after briefly discussing the MRI technique, we review the MRI appearances of various local complications of acute pancreatitis, ranging from relatively common necrotic and hemorrhagic conditions to rare conditions, such as peripancreatic abscess and pseudoaneurysm. Knowledge of the classic MRI findings of these complications allows prompt recognition of the related pathologic condition, and might influence the choice of treatment.

MRI TECHNIQUE

For the appropriate evaluation of local complications of acute pancreatitis, it is necessary to assess all parts of the pancreas, including the peripancreatic parenchyma and the vasculature[1]. The general MRI sequences for pancreatitis require the combined use of T1-weighted sequences [e.g. fast spin-echo (FSE) imaging with multiple breath-hold acquisitions or single-breath-hold gradient echo (GRE) imaging], T2-weighted sequences [e.g. fast recovery fast spin-echo (FRFSE) or single-shot fast spin-echo (SSFSE) imaging], and MRCP (e.g. a thick-slab, SSFSE MRCP). The advantages or limitations of these sequences are as follows: (1) T1-weighted scan with fat suppression improves the delineation of pancreatic borders and the pancreas itself, and it has important value in MRI acquisition for evaluation of hemorrhagic complications of acute pancreatitis[3]. However, T1-weighted sequences with breath holding require patient cooperation, otherwise, respiratory artifacts will occur, which might affect the visualization of the whole pancreas and its adjacent structure[1-3]; (2) T2-weighted sequences have significant advantages in demonstrating fluid-filled lesions in or around the pancreas and the pancreatic duct, and SSFSE T2-weighted sequence can be used to guide acquisition of an MRCP series[2,3]. Using respiratory triggered or navigator-echo-based techniques allows the investigating of less cooperative patients; and (3) MRCP is obtained before gadolinium administration, allows noninvasive evaluation of pancreatic ducts and the whole extrahepatic biliary tract, and provides few respiratory artifacts or susceptibility effects[1-3]. However, the main weakness of MRCP for ductal visibility is the potential overlap of other fluid-containing organs (e.g. the stomach and duodenum)[3].

The methodology of contrast-enhanced MRI for patients with acute pancreatitis in our institute is detailed as follows. A 19-gauge needle was placed in the right antecubital vein. A dose of 0.2 mL/kg Gd-DTPA (469.01 mg/mL Bayer Schering Pharma AG; Berlin, Germany) was then administered. A bolus of 20 mL of physiological saline was given immediately following gadolinium administration. The contrast agent and physiological saline were injected at an infusion rate of 3 mL/s with a power injector (Spectris MR Injector System; Medrad, USA). After gadolinium chelate injection, the optimal dynamic contrast-enhanced method [e.g. T1-weighted acquisition performed with liver acquisition with volume acceleration (LAVA)] was used to assess the extent of pancreatic necrosis for the initial staging of acute pancreatitis, which has been shown to correlate with the course of the disease[1-3]. MRI LAVA scanning starts at 14-16 s after the intravenous administration of gadolinium. It costs a total of 65 s for three-phase dynamic contrast-enhanced scans (15 s for each phase and 10 s of the interval of phases for breathing). A total of (80-112) × 3 contrast-enhanced frames were obtained. Moreover, MRA, the post-processing technique after MRI LAVA, can be performed to supplement the information for visualization of pancreatic vascular network

Table 1 MRI parameters

Sequence	TR (ms)	TE (ms)	Slice thickness (mm)	Slice space (mm)	Matrix	Field of view (mm)	Number of excitation	Flip angle
FSE T1W	300	10	5	0.5-1	256 × 192	380 × 340	2	90°
GRE T1W	195	1.5	5-7	0.5-1	256 × 192	360 × 340	1	70°
FRFSE T2W	8000-13500	90-120	5	0.5-1	256 × 224	400 × 340	1	90°
SSFSE T2W	Not applicable	80-197	5	0.5	256 × 192	390 × 330	1	90°
SSFSE MRCP	Not applicable	830-1100	40-50	0	256 × 192	320 × 240	2	90°
LAVA T1W	3.8-6.1	1.8-2.1	5[1]	0	256 × 224	380 × 340	1	15°-20°

[1]A section thickness of 5 mm for LAVA is the section thickness of automatic reconstruction images from this sequence. MRI: Magnetic resonance imaging; FSE: Fast spin-echo; GRE: Gradient echo; FRFSE: Fast recovery fast spin-echo; SSFSE: Single-shot fast spin-echo; LAVA: Liver acquisition with volume acceleration; T1W: T1-weighted; T2W: T2-weighted.

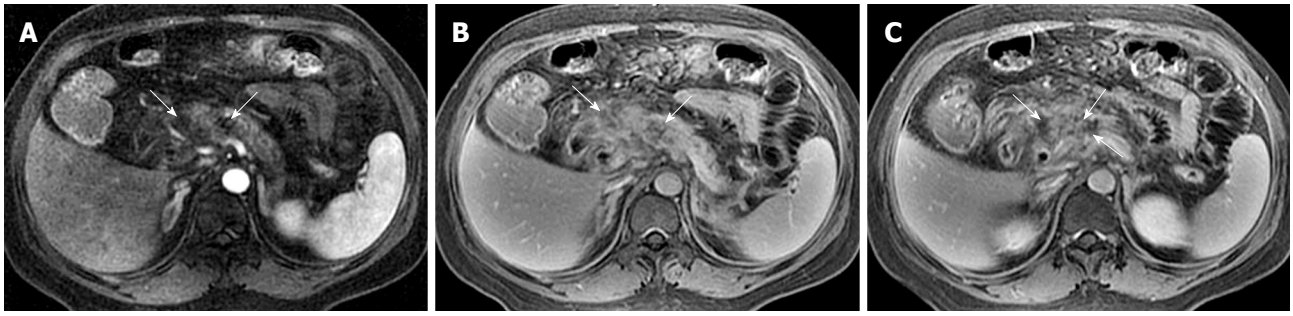


Figure 1 43-year-old woman with acute pancreatitis. Contrast-enhanced axial T1-weighted images during arterial phase (A) and late venous phase (B, C) show spotted, patchy non-enhanced areas in the pancreatic head and body (arrows).

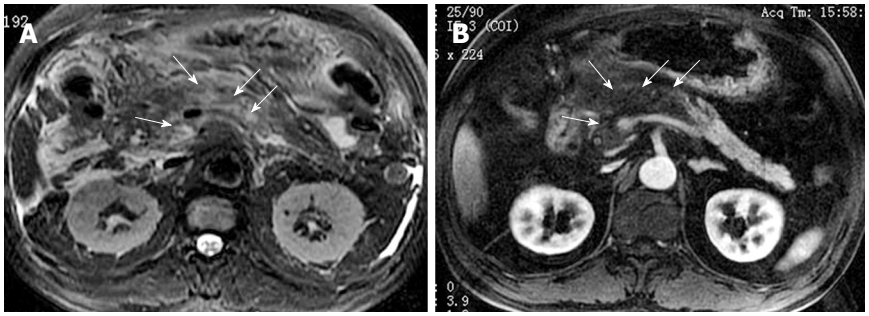


Figure 2 65-year-old man with acute pancreatitis. Axial T2-weighted image (A) shows the high-signal intensity in the pancreatic head and body (arrows). The arterial phase MR image (B) shows a large necrotic area ("black pancreas") in the pancreatic head and body (arrows).

and vascular complications of acute pancreatitis. The detailed protocols used with our 1.5-T MR imager for these sequences are listed in Table 1.

MRI FINDINGS

Necrosis

Pancreatic necrosis refers to a pathological collection of devitalized tissue in the pancreas, which is a relatively common local complication of acute pancreatitis[10].

Necrosis is diagnosed when focal or diffuse, conspicuous hypointense areas on T1WI corresponding to hyperintense areas on T2WI and well-marginated areas of non-enhancing pancreatic parenchyma in comparison to the signal intensity of the normal pancreatic parenchyma are present[1-3]. Dynamic contrast-enhanced MRI examination can identify surviving pancreatic tissue[10]. The focal pancreatic necrosis is characterized by multiple spotted, patchy non-enhancing areas (like "pepper") on contrast-enhanced MR images (Figure 1). The large diffuse non-enhanced area of the pancreas (the so-called "black pancreas") in acute pancreatitis on enhanced MR images represents diffuse pancreatic necrosis (Figure 2).

Necrosis of peripancreatic fatty tissue, which is a form of inflammatory extension, involves the spread of peripancreatic intra-abdominal fatty tissue and adipose tissue in the retroperitoneal space[2]. The covering of the pancreas is surrounded by the formation of the capsule, which is only a series of loose connective tissue. Therefore, the pancreatic capsule forms almost no block on the role of inflammatory extension. MRI might show the intra-abdominal inflammatory involvement located in omental or mesenteric fatty contents regions (Figure 3). The affected retroperitoneal space manifests the inflammation extension from the retroperitoneal space to anterior pararenal space of the left kidney, which is complicated by retroperitoneal steatonecrosis (Figure 4)[2,3]. It is important and helpful in evaluating the prognosis of acute pancre-

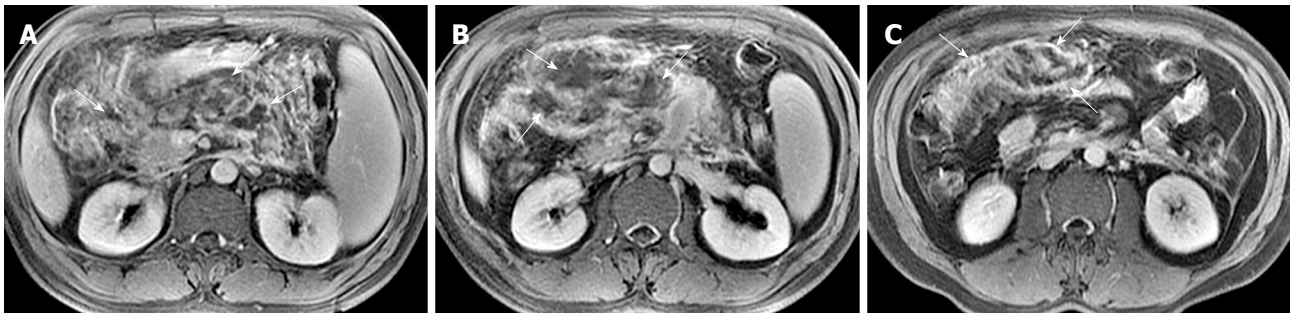


Figure 3 34-year-old man with acute pancreatitis. Contrast-enhanced axial T1-weighted images show peripancreatic inflammation extension (arrows) along the mesenteric adipose tissue region (A, B). There is addition irregular thickening and enhancement of the adjacent intestinal wall (arrows, C).

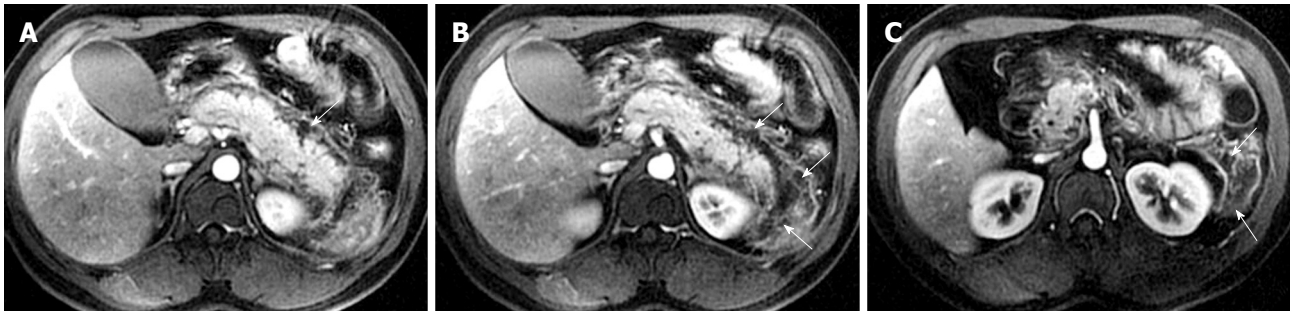


Figure 4 31-year-old man with acute pancreatitis. Contrast-enhanced axial T1-weighted images with fat suppression reveal thickening and enhancement of dorsal peritoneum (arrow, A). The peripancreatic inflammation extended from the retroperitoneal space to the anterior pararenal space of the left kidney (arrows, B and C).



Figure 5 30-year-old man with acute pancreatitis. Unenhanced axial T1-weighted image with fat suppression shows patchy hemorrhagic foci (arrows) in the pancreas and in the fatty tissue behind the pancreas.

atitis to differentiate between necrosis of peripancreatic tissue and central pancreas necrosis. Neoptolemos *et al*[11] reported that the evaluation of the integrity of the main pancreatic duct might be helpful in making a distinction between mild pancreatitis and severe pancreatitis, and for determining the necessity for surgery for local pancreatic complications in severe pancreatitis.

Hemorrhage

Hemorrhage of the pancreas is seen in 2%-5% of patients with acute pancreatitis and might occur in the setting of severe necrotizing pancreatitis[10]. With conversion from hemoglobin to methemoglobin in the hemorrhage regions, MRI findings of hemorrhagic lesions show spotted or patchy high signal intensity (like "salt") in T1-weighted images with fat suppression (Figure 5).

Peripancreatic hemorrhage might be secondary to the necrosis of peripancreatic tissue, which is visualized as patchy or large diffuse areas with hyperintensity on T1-weighted images with fat suppression[2] (Figure 6). Martin *et al*[12] reported that elevated peripancreatic signal on fat-suppressed T1-weighted spoiled gradient echo images is associated with poor outcome in acute pancreatitis. This might represent a simplified imaging method in regards to its relationship to clinical prognosis. Other local complications of acute pancreatitis, such as pancreatic pseudocysts and peripancreatic vascular invasion, can also show hemorrhage (mentioned below).

Infection

Pancreatic cellulitis is an infectious complication in patients with acute pancreatitis, whose incidence varies from 8.3% to 10.6%, and can be difficult to differentiate clinically from pancreatic abscess[13,14]. It is a clinical or radiological entity in the pancreas and peripancreatic region as a result of pathological pancreatic swelling, inflammatory cell infiltration, and tissue necrosis[15]. Contrast-enhanced MRI examination can show the range of the involvement of pancreatic cellulitis. MRI findings of pancreatic cellulitis include the formation of an ill-defined mass with ring-like and separated enhancement (like "Hornet's nest"), and non-enhanced regions within the central part of the mass (Figure 7). Pancreatic cellulitis can be absorbed completely and might also progress to a pancreatic abscess.

Pancreatic abscess is a typical infectious manifestation of acute necrotizing pancreatitis, whose incidence varies from 1% to 9%, and can result in high mortality in acute

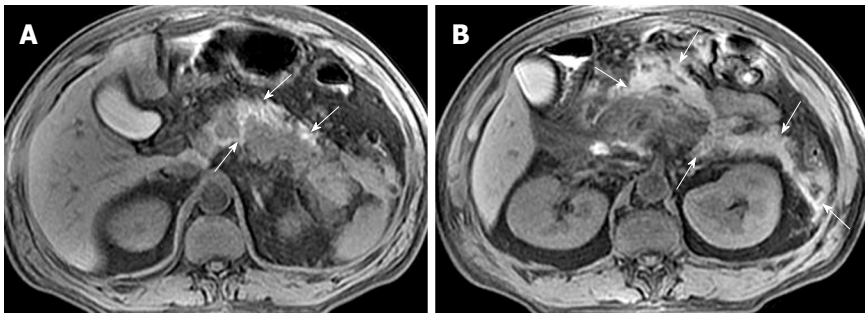


Figure 6 50-year-old man with acute pancreatitis. Unenhanced axial T1-weighted images with fat suppression reveal pancreatic and peripancreatic hemorrhage (arrows, A and B); Peripancreatic hemorrhage involvement include the retroperitoneal space and anterior pararenal space of the left kidney (arrows, B).

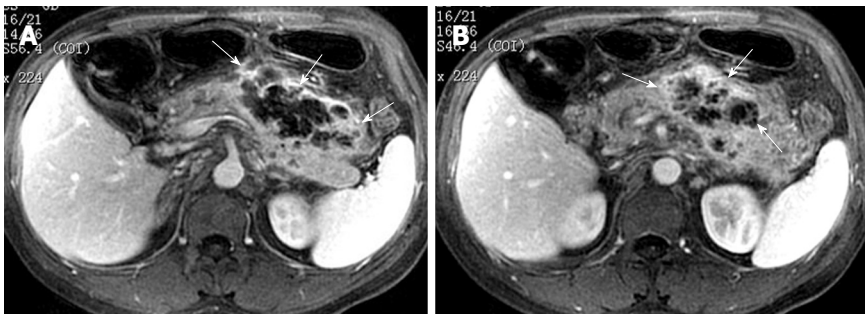


Figure 7 39-year-old man with acute necrotizing pancreatitis and peripancreatic cellulitis. Contrast-enhanced T1-weighted images show a ring-like and separated enhancing mass in pancreatic adjacent retroperitoneal space (arrows, A and B).

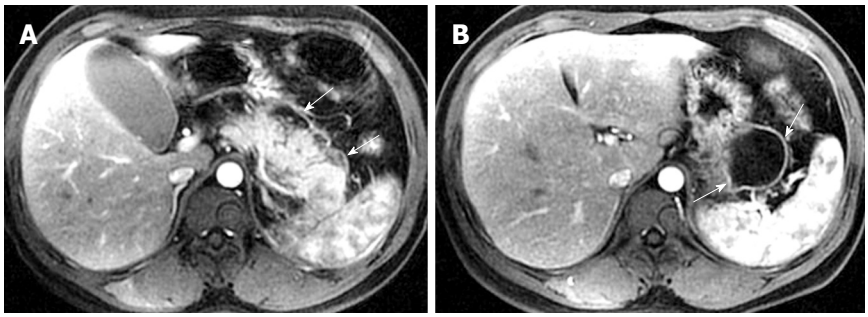


Figure 8 33-year-old man with acute necrotizing pancreatitis and peripancreatic abscess. Contrast-enhanced axial T1-weighted images reveal abnormal peripancreatic enhancement (arrows, A) and a liquid-like mass with a well-defined, thickened, and enhancing wall (arrows, B).

pancreatitis patients[16]. If pancreatic cellulitis was not well controlled, abscesses might occur 3-5 wk after the onset of acute pancreatitis and might be similar to pseudocysts[10]. Pancreatic abscesses with acute pancreatitis induce a systemic infection poisoning reaction resulting in symptoms of fever and local peritonitis. The performance of a pancreatic abscess on MRI might present as an encapsulated liquid-form mass with a significantly enhancing wall (Figure 8).

Pseudocyst

Pancreatic pseudocysts are local complications of acute or chronic pancreatitis, and they can be classified as acute pseudocysts in patients suffering from acute post-necrotic pancreatitis, and chronic pseudocysts that develop in patients already suffering from chronic pancreatitis[17]. The pathogenesis of pancreatic pseudocysts might be associated with an actively secreting pancreas in acute or chronic pancreatitis; an encapsulated cyst communicating with pancreatic ducts and/or partial pancreatic ductal obstruction. Patients with pancreatic pseudocysts are treated conservatively or percutaneously with aspiration under ultrasound guidance or surgically with external catheter drainage[17].

Pancreatic pseudocysts might be located within the pancreatic tissue or surrounding the pancreas. They often occur approximately four weeks after a first episode of acute pancreatitis[18-20]. MRI findings of a simple pseudocyst include a round or oval fluid collection surrounded by a thin or thick wall, with liquid signal performance of hypointensity on T1-weighted images and hyperintensity on T2-weighted images (Figure 9). Pancreatic pseudocysts can also present as complex pseudocysts associated with mucus, protein, and hemorrhage[2]. This often manifests as a heterogeneous lesion dominated by hyperintensity on T1-weighted images with fat suppression (Figure 10). Therefore, compared with CT, MRI might be more favorable when the a suspected pseudocyst with bleeding occurs.

Vascular disorders

Vascular complications of acute pancreatitis represent a spectrum of vascular abnormalities, such as peripancreatic artery involvement including pseudoaneurysm, and vein invasion including phlebothrombosis[1-4]. Dörffel *et al*[21] assessed vascular complications in acute pancreatitis by color duplex ultrasonography: the incidence of arterial pseudoaneurysms was 7% in acute pancreatitis, and the incidence of venous thromboses was 30% in acute non-necrotizing pancreatitis and 57% in necrotizing pancreatitis. MRI findings of artery invasion consist of the loss of normal vascular flowing void effect, the blurred edge of the local involving arterial wall and the

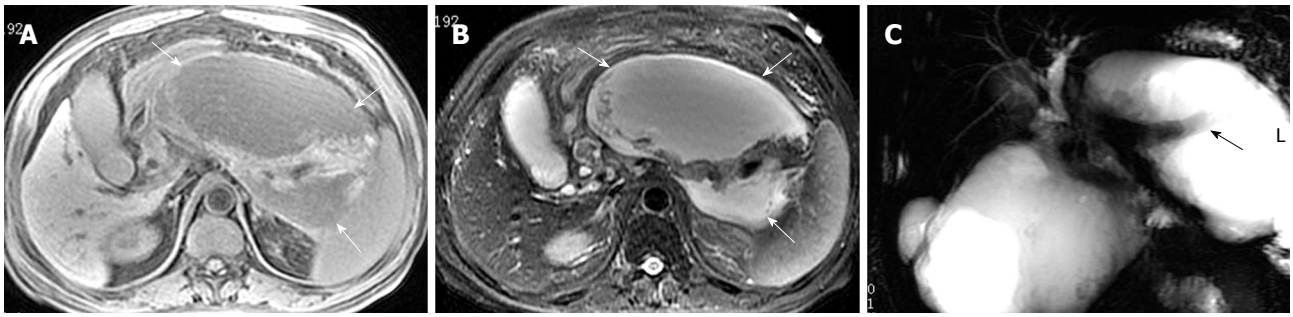


Figure 9 41-year-old man with a large peripancreatic pseudocyst who presented as abdominal distension for follow-up after acute pancreatitis. Unenhanced axial T1-weighted (A) and axial T2-weighted (B) images show a large lesion with typical liquid signal intensity around the pancreas (arrows). MRCP image (C) reveals the pancreas wrapped inside the large pseudocyst, just like the swimming fish (arrow).



Figure 10 35-year-old man with multiple pseudocysts complicated hemorrhage. Patient had previously suffered from acute pancreatitis. Unenhanced axial T1-weighted images (A and B) with fat suppression and axial T2-weighted image (C) with fat suppression reveal hemorrhagic pseudocysts with a mixed signal intensity dominated by hyperintensity (arrows).



Figure 11 30-year-old man with acute pancreatitis and splenomegaly. Axial T2-weighted images (A and B) show the involved segments (arrows) of splenic artery (A) and splenic vein (B). Enhanced arterial phase (C) and venous phase (D) images reveal clearly the involved segments (arrows) of the splenic artery (C) and vein (D).

poor enhancement at the involving segment on contrast-enhanced arterial phase images (Figure 11). The splenic artery, gastroduodenal artery, and pancreaticoduodenal artery are more frequently involved[10,22].

Pseudoaneurysm is an uncommon, usually delayed, complication of acute pancreatitis and might constitute a life-threatening emergency if rupture occurs[10]. MRI can directly show the pseudoaneurysm cavity that communicates with the adjoining arteries and the mural thrombosis in the pseudoaneurysm cavity. On enhanced MR images, the enhancement of pseudoaneurysm cavity corresponds with the arteries, whereas the non-enhanced region is illustrated as the mural thrombus (resembling a Chinese "Yin-Yang" diagram)[23] (Figure 12).

The splenic vein is the most common vein invaded by pancreatic inflammatory extensions due to its prox-

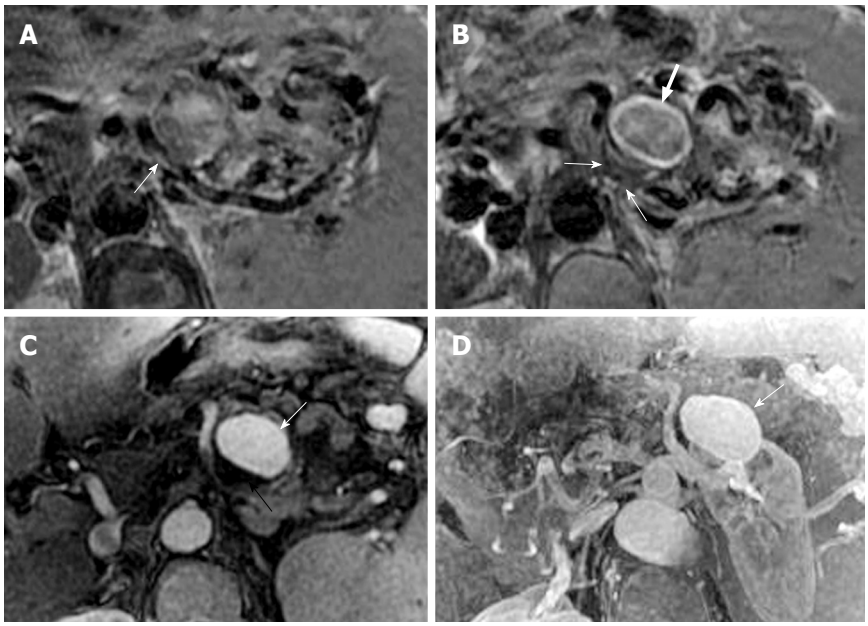


Figure 12 36-year-old man with a history of acute pancreatitis and a splenic artery pseudoaneurysm. The involved segment of the splenic artery (arrow, A) and an aneurysmal dilatation structure (large arrow, B) are seen on axial T2-weighted images. On enhanced arterial phase image (C), the enhancement of the pseudoaneurysm cavity (white arrow) and the crescent-form filling defect (black arrow) are similar to the Chinese "Yin-Yang" diagram. This pseudoaneurysm is also illustrated on contrast-enhanced MR angiography (arrow, D).

imity to the pancreas[10,22]. The involving vein segment might be complicated by local venous thrombosis. MRI shows the loss of normal vascular flowing void effect at this involving vein segment. After administration of a contrast agent, an intravenous filling defect can be seen on enhanced venous phase images (Figure 11).

Practical setup of MRI in local complications of acute pancreatitis

Cross-sectional T2-weighted imaging and contrast-enhanced fat-suppressed T1-weighted imaging combined with MRCP can be used to assess the severity of acute pancreatitis before therapeutic planning[1-3]. However, dynamic contrast-enhanced MRI in acute pancreatitis is not routinely performed, because, for the majority of patients, pancreatic change is easy to establish with various T1-weighted and T2-weighted sequences in transverse and coronal sections. Therefore, in our current pancreatic imaging practice, the decision process as to whether patients receive dynamic studies is as follows: (1) For imaging of pancreatic necrosis, the combination of T1-weighted and T2-weighted findings with dynamic contrast-enhanced appearances represents a comprehensive evaluation of the extent of necrosis and full range of inflammatory extension. We recommend that enhanced-MRI is essential; (2) For imaging of infectious complications, dynamic contrast-enhanced examinations might help differentiate pancreatic cellulitis or abscesses from pancreatic fluid collection or simple pseudocysts; and (3) For imaging of vascular abnormalities, the combination of cross-sectional pancreatic parenchyma imaging with MRA offers the possibility of a single diagnostic modality for the full evaluation of peripancreatic artery and vein involvement, such as arterial pseudoaneurysms and venous thromboses.

CONCLUSION

In summary, MRI might be indicated in acute pancreatitis for detecting and characterizing local complications of acute pancreatitis. Enhanced-MRI is essential for a comprehensive evaluation of the extent of pancreatic necrosis.

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ORIGINAL ARTICLE

Unlocking the ultrastructure of colorectal cancer cells *in vitro* using selective staining

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Author contributions: Biazik JM prepared the manuscript for publication, conducted the selective staining and sectioning for transmission electron microscopy, data analysis, and data interpretation for the three cell lines; Jahn KA, Su Y and Wu YN carried out cell culture on the three cell lines; Braet F assisted in data interpretation and manuscript preparation.

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Abstract

AIM: To characterise differences between three widely used colorectal cancer cell lines using ultrastructural selective staining for glycogen to determine variation in metastatic properties.

METHODS: Transmission electron microscopy was used in this investigation to help identify intracellular structures and morphological features which are precursors of tumor invasion. In addition to morphological markers, we used selective staining of glycogen as a marker for neoplastic cellular proliferation and determined whether levels of glycogen change between the three different cell lines.

RESULTS: Ultrastructural analysis revealed morphological differences between the cell lines, as well as differentiation into two sub-populations within each cell line. Caco-2 cells contained large glycogen deposits as well as showing the most obvious morphological changes between the two sub-populations. SW480 cells also contained large glycogen stores as well as deep cellular protrusions when grown on porous filter membranes. HT-29 cells had trace amounts of glycogen stores with few cellular projections into the filter pores and no tight junction formation.

CONCLUSION: Morphology indicative of metastatic properties coincided with larger glycogen deposits, providing strong evidence for the use of selective staining to determine the neoplastic properties of cells.

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Key words: Cancer; Colorectal; Electron microscopy; Glycogen; Potassium ferrocyanide

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INTRODUCTION

Cultured human colon carcinoma Caco-2, HT-29, and SW480 cell lines have been used as model cell lines by many researchers to investigate the cell biology and metastatic properties of colorectal cancer. The main tissue of origin of these cell lines, the colon, is a structure comprising a dynamic epithelium where there is continuous

renewal of normal colonic epithelial cells that originate in the crypts (secretory cells) and migrate to the tips of the villous folds (absorptive cells)[1]. This, therefore, results in cellular heterogeneity of all epithelial cells that are derived from any human adenocarcinoma cell line. The present study is designed to examine these heterogenous features ultrastructurally within the three chosen cells lines to determine whether varying malignant characteristics are expressed.

All epithelia form a barrier made of polarized cells in which the apical and basolateral domains of the cells can be separated into functional domains[2]. Cell adhesion is critical in the organisation of normal epithelia, in particular the establishment of the adherens junction, mediated by the presence of E-cadherin, which initiates a cascade of events leading to the formation of the tight junction, gap junction, and desmosomes, all key regulators of the junctional complex[3-5] and mediators of cellular polarisation. The loss of these structures results in the breakdown of the epithelium, leading to increased permeability and pathogenesis of disease[4]. An ultrastructural investigation into the various features of the junctional complex will provide evidence of cellular communication and adhesion to determine whether cellular polarisation is present.

Additionally, the invasive properties of cells are also reflected by cell shape, whereby epithelioid cells appear to be less invasive than spindle-shaped cells[6] due to cellular polarisation and establishment of a tight junction. Additional ultrastructural assays that might help determine the differences in the invasive properties of the aforementioned cells lines is their ability to leave the compartment to which they are normally restricted and to determine whether cells have the potential to carry out invasive growth. By culturing these cells on porous membrane filters, cellular anchorage and cellular sensing movement can be investigated by analysing the degree of cellular migration into the filter pores. By examining these characteristics at an ultrastructural level, comparative invasive properties can be evaluated in each of the cell lines.

In addition to using cellular morphology as a marker for invasive properties, storage of glycogen will also be analysed. Functionally, glycogen storage in the epithelium provides an energy store for cellular proliferation[7-9]. Large quantities of glycogen stores have also been reported in a variety of cultured cells, in particular cells undergoing neoplastic transformations[9-11]. Glycogen metabolism has been widely investigated in malignant cells originating from tissues which normally store glycogen, i.e. hepatomas[11,12] and cancers of the cervix[13]. Surprisingly, elevated stores of glycogen have been reported in all malignant cell lines (in a 58 cell line culture study) irrespective of tissue of origin[9], and in particular colon adenocarcinomas, originating from human colonocytes where glycogen is normally absent[9,10]. Furthermore, the variation in the amount of glycogen storage has been utilized as a marker for malignant processes in cells, whereby increasing levels of glycogen are indicative of more malignant cells due to abnormal cellular proliferation[9]. Glycogen storage has been reported in Caco-2, HT-29, and SW480 cells *via* a glycogen quantitative assay. The highest glycogen levels were found in Caco-2 cells, intermediate levels in HT-29 cells, and the lowest levels in SW480[10], suggesting that Caco-2 cells undergo extensive abnormal cellular proliferation compared to HT-29 and SW480 cells. These assays have not, however, yielded any information on the ultrastructural position of glycogen in the cells, as accumulation of glycogen particles are first observed in the apical cytoplasm followed by a basal distribution when glycogen particles continue to accumulate[7]. By incorporating a selective staining method to detect the presence of glycogen in the cell, this investigation will contribute to the ultrastructural localization of glycogen particles in these colorectal cancer cell lines for the evaluation of malignant properties.

Osmium tetroxide has been widely used by electron microscopists to routinely fix and stain intercellular membrane systems[14,15]; however, primary fixation in an osmium tetroxide-potassium ferrocyanide [$K_4Fe(CN)_6$] mixture combines selective fixation and staining of various cellular components, in particular glycogen[16,17] and intracellular membranes[14,18]. Osmium-ferrocyanide has been shown to bind selectively through the C_2 and C_3 dihydroxyl groups in glycogen. Chelation of the osmium-ferrocyanide complexes by donor atoms in the tissue helps reduce the osmium to a more stable oxidation state, allowing greater osmium deposition than that observed through post-fixation with osmium tetroxide alone. Therefore the osmium-ferrocyanide complex provides excellent preservation and contrast of glycogen complexes, which in many cases might otherwise remain unstained[17]. The osmium-ferrocyanide method will be incorporated into the present study to determine whether there is an ultrastructural variation of glycogen distribution between different colorectal cancer cell lines.

This study will use three cell lines: Caco-2, HT-29 and SW480 cells. The human colon adenocarcinoma cell line, Caco-2, undergoes spontaneous differentiation in cell culture resulting in structural and functional characteristics which resembles intestinal epithelium[19-23]. The Caco-2 cell line, although derived from a tumor of the human colon epithelium (colonocytes), surprisingly exhibits characteristics of foetal ileal epithelial cells from the small intestine (enterocytes)[23]. Many reports have identified the formation of a heterogenous population of Caco-2 cells in culture[7,20,24-26]. One population consists of dome-forming simple columnar polarized epithelium with a homogenous distribution of microvilli (brush border)[27] and established junctional complexes, in particular the tight junction[4,5]. The variant population comprises multilayered cuboidal cells[28], exhibiting large intercellular spaces (cysts) characteristic of motile cells[24,29]. Due to this functional differentiation, Caco-2 cells provide a model to investigate the intestinal barrier as well as drug transport across the intestinal mucosa[19,22] and provide our study with a model cell line that exhibits a heterogenous cell population.

HT-29 cells, although not homogenous in nature, when grown in control Dulbecco's modified Eagle's medium (DMEM) remain morphologically undifferentiated and comprise of a multilayer of unpolarized cells

that contain features of mucous secreting cells[30-32]. The junctional complex and, in particular, the tight junction is absent[33], resulting in disorderly development of cells. A unique feature of HT-29 cells is their ability to undergo polarisation and reversible differentiation into enterocytes after administration of galactose into the culture medium[31]. The undifferentiated form of HT-29 cells will be analysed in this investigation to avoid confounding effects attributable to cell culture medium to allow for a comparative framework between cells lines to investigate junctional properties, glycogen content, and other microanatomy of the HT-29 cell line.

The SW480 cell line also consists of a heterogenous population of cells, but these cells do not differentiate[34]. The predominant cell type in the SW480 cell line consist of flat polygonal cells which form typical epithelial colonies (E-type); the variant R-type cells consist of round, refractile cells with a disoriented morphology. The R-type cells display anchorage-independent growth, form multilayers and are regarded as more malignant[35]. Cellular morphology, glycogen content, and desmosome formation to determine cellular contact and organisation will be used to identify whether the predominant population of cells in the investigation consist of the E- or R-type.

The comparative ultrastructural and glycogen storage data will be examined in the Caco-2, HT-29 and SW480 cell lines at day 1 and day 12 post-confluency to help promote cellular differentiation. The data collected from this investigation will contribute to the utilisation of microanatomical cellular features to determine neoplastic transformations in human adenocarcinoma cell lines and to help identify sub-populations in the chosen cell lines.

MATERIALS AND METHODS

Cell culture

Caco-2, HT-29, and SW480 cell lines were obtained from the American Type Culture Collection. Caco-2 and HT-29 cells were cultured in advanced Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal calf serum, 0.5 mL Glutamax, and 0.5 mL antibiotics per 40 mL of medium. SW480 cells were cultured in L15 medium supplemented with 10% fetal calf serum, 0.5 mL Glutamax, and 0.5 mL antibiotics per 40 mL of medium. The cells were incubated at 37℃ in a humidified incubator with 5% CO_2 in air and medium was changed every day after the cells reached confluency.

Transmission electron microscopy

Cells were cultured on 0.4 μm pore size cell culture inserts (BD Falcon, NSW Australia) and collected on day 1 and day 12 post-confluency. Four culture inserts per treatment day were fixed in 2.5% gluteraldehyde (ProSci Tech, Queensland, Australia) in 0.1 mol/L sodium cacodylate buffer with 0.1 mol/L sucrose pH 7.4 for 1 h. Cells were next postfixed for 1 h in a 1% osmium tetroxide solution containing 0.8% potassium ferrocyanide in 0.1 sodium cacodylate buffer (to enhance plasma membranes and glycogen particles), dehydrated in graded alcohols, and infiltrated with 50% Spurr's resin/absolute ethanol for 1 h. The cells were then re-embedded in fresh Spurr's resin (Agar Scientific, Essex, UK) overnight under gentle agitation, re-embedded in fresh Spurr's resin, and left to polymerized at 60℃ for 24 h. Two areas of each cell culture insert (n = 8) were cut using a Leica ultracut UCT ultramicrotome (Leica, Heerbrugg, Switzerland) and silver-gold sections (80-90 nm) were mounted onto 200-mesh copper grids. Sections were stained with a 2% solution of aqueous uranyl acetate for 10 min, followed by Reynold's lead citrate stain for 10 min. Stained sections were then viewed using a JEOL 1400 (Tokyo, Japan) transmission electron microscope operating at 120 kV.

RESULTS

Overall, the application of the potassium-ferrocyanide methods results in selective staining of glycogen particles (approximate 20 nm in diameter), electron dense demarcation of plasma membranes, and demarcation of intranuclear indentations (intranuclear rods).

Ultrastructure of Caco-2

When grown on membrane filters, Caco-2 cells differentiated into two morphologically different populations, which will be described separately (Table 1).

Sub-population 1

On day 1 post-confluency, cells were simple squamous with irregular sparse microvilli (Figure 1A). Cells contained round euchromatic nuclei and showed evidence of all other cellular organelles, including mitochondria, rER, and Golgi (Figure 1B). Intercellular spaces between cells were evident, as well as tight junctions and desmosomes. When grown on porous membrane filters (Figure 1C), cellular processes marginally invaded into filter pores (Figure 1D). On day 12 post-confluency, cellular morphology and organisation changed to a stratified cuboidal formation and intercellular spaces increased, resulting in the loss of the tight junction, yet desmosomes were still evident (Figure 1E and F). Glycogen granules were distributed within the cytoplasm with a supranuclear predisposition (Figure 1E and G). Extensive intranuclear rods (indentations of the nuclear membrane) were evident (Figure 1F) and no invasive processes were observed projecting into the filter pores (Figure 1H).

Sub-population 2

On day 1 post-confluency, cells were simple cuboidal and contained regular microvilli (Figure 2A). No intercellular spaces were evident and a tight junction was present along the apical region of the lateral plasma membrane (Figure 2B). Small amounts of glycogen particles were evident and regularly distributed (Figure 2C). Several cellular processes projected into the filter pores (Figure 2D). On day 12 post-confluency, cells became simple columnar with a homogenous population of microvilli (Figure 1E). Glycogen accumulation increased in the cytoplasm of

Table 1 Ultrastructural and morphological features of Caco-2 (sub-population 1 and 2, HT-29 and SW480 cell lines)

Cell line	Days post-confluency	Cell type	Nucleus	Intercellular space	Microvilli	Invasive processes	Mitoch-ondria	Golgi	sER	rER	Junctions	Glycogen	Vacuoles
Caco-2 sub-pop 1	1	Simple squamous	Indented (I/N rods)	++	+/-	+/-	+	+	+	+	+Des -TJ	-	-
Caco-2 sub-pop 1	12	Stratified cuboidal	I/N rods	+++	+	+/-	+	+	+	+	+Des -TJ	+++	-
Caco-2 sub-pop 2	1	Simple squamous	Round	+	++	+/-	+	++	+	+	++Des +TJ	-	+
Caco-2 sub-pop 2	12	Simple columnar	Round	+	+++	-	++	++	+	++	+++Des +TJ	+++	+
HT29	1	Stratified cuboidal	Round	+++	+++	-	+	+	+	+	++Des -TJ	-	-
HT29	12	Stratified cuboidal	I/N rods	+++	+++	-	+	+	+	+++	++Des -TJ	+/-	+++
SW480	1	Simple squamous	Round	+	+++	+++ 4 μm	+++	++	+	+++	+/-Des -TJ	-	-
SW480	12	Simple squamous (spindle shape)	Round	+	+++	+++ 8 μm	+++	++	+	+++	+/-Des -TJ	+	-

-: Absent; +/-: Present; +: Small; ++: Intermediate; +++: Large; I/N: Intranuclear; Des: Desmosomes; TJ: Tight junction.

these cells, exhibiting a supra- and sub-nuclear distribution (Figure 2F). Mucous storing vacuoles also occupied a large volume of these cells and were located in close proximity of the glycogen particles. Intercellular spaces were evident in the basal region of the lateral plasma membrane and the tight junction and plasma membrane interdigitation was also present (Figure 2F). No invasive projections into the filter pores were evident (Figure 2H).

Ultrastructure of HT-29 cells

No morphological heterogeneity was observed in the HT-29 cell line.

On day 1 post-confluency, the cells were stratified cuboidal with microvilli projecting into large intercellular spaces between cells (Figure 3A and B). Desmosomes were evident at meeting points along the lateral plasma membrane (Figure 3C and D). The nuclei were round and euchromatic. Mitochondria, rER and Golgi bodies were all present. The apical-most cells of the multilayer contained regular microvilli; no invasive processes were present and there were no obvious glycogen stores. On day 12 post-confluency, cells remained stratified cuboidal, expressing large intercellular spaces with microvilli projecting into them (Figure 3E). Golgi bodies were more evident, as well as large accumulations of mucous secreting vacuoles (Figure 3F and G). Desmosomes were present along contact sites of the lateral plasma membrane (Figure 3G) and no significant invasive processes or glycogen stores were evident (Figure 3H).

Ultrastructure of SW480 cells

On day 1 post-confluency, cells were simple squamous with irregular microvilli (Figure 4A). Mitochondria were densely distributed through the cell cytoplasm (Figure 4B) and tight junction formation was evident when upper leaflets of the plasma membrane were in close contact (Figure 4C). Long invasive cellular projections were present in the filter pores (Figure 4D). On day 12 post-confluency, cells were simple cuboidal with irregular sparse microvilli (Figure 4E). Large numbers of mitochondria with filamentous branching were evident, as well as the presence of glycogen accumulation (Figure 4F and G). Small intercellular spaces were present, but no desmosomes were evident (Figure 4F). Extremely long invasive processes projected into the filter pores and these projections contained cellular organelles, such as mitochondria (Figure 4H).

DISCUSSION

Utilising selective staining to examine differences in metastatic properties of Caco-2, HT-29, and SW480 cells advances our understanding of the commonly used colorectal cancer cell lines. Morphologically, after reaching confluency, many differences were apparent between the cell lines in this investigation. When grown in standard culture conditions, spontaneous differentiation predominantly occurred in the Caco-2 cell line; however, ultrastructural differences suggesting heterogenous populations also became evident in the HT-29 and SW480 cell lines when cells were examined 12 d post-confluency.

Evidence of a heterogenous population already existed in the Caco-2 cells one day after reaching confluency. Although both Caco-2 cell populations were simple squamous, one population exhibited regular microvilli and tight junction formation (sub-population 2), whereas the other population was void of these features (sub-population 1). By day 12 post-confluency, this heterogeneity was even more apparent, as population 2 cells exhibited a tight junction and became a simple layer of columnar polarized cells with regular microvilli indicative of regular epithelial cell assembly. No intercellular spaces were evident between adjoining cells and there was no evidence of invasive projections into membrane pores; glycogen accumulation, however, was abundant. These features were indicative of intestinal epithelial cells[19,20] and suggested that this sub-population of cells originated from the absorptive

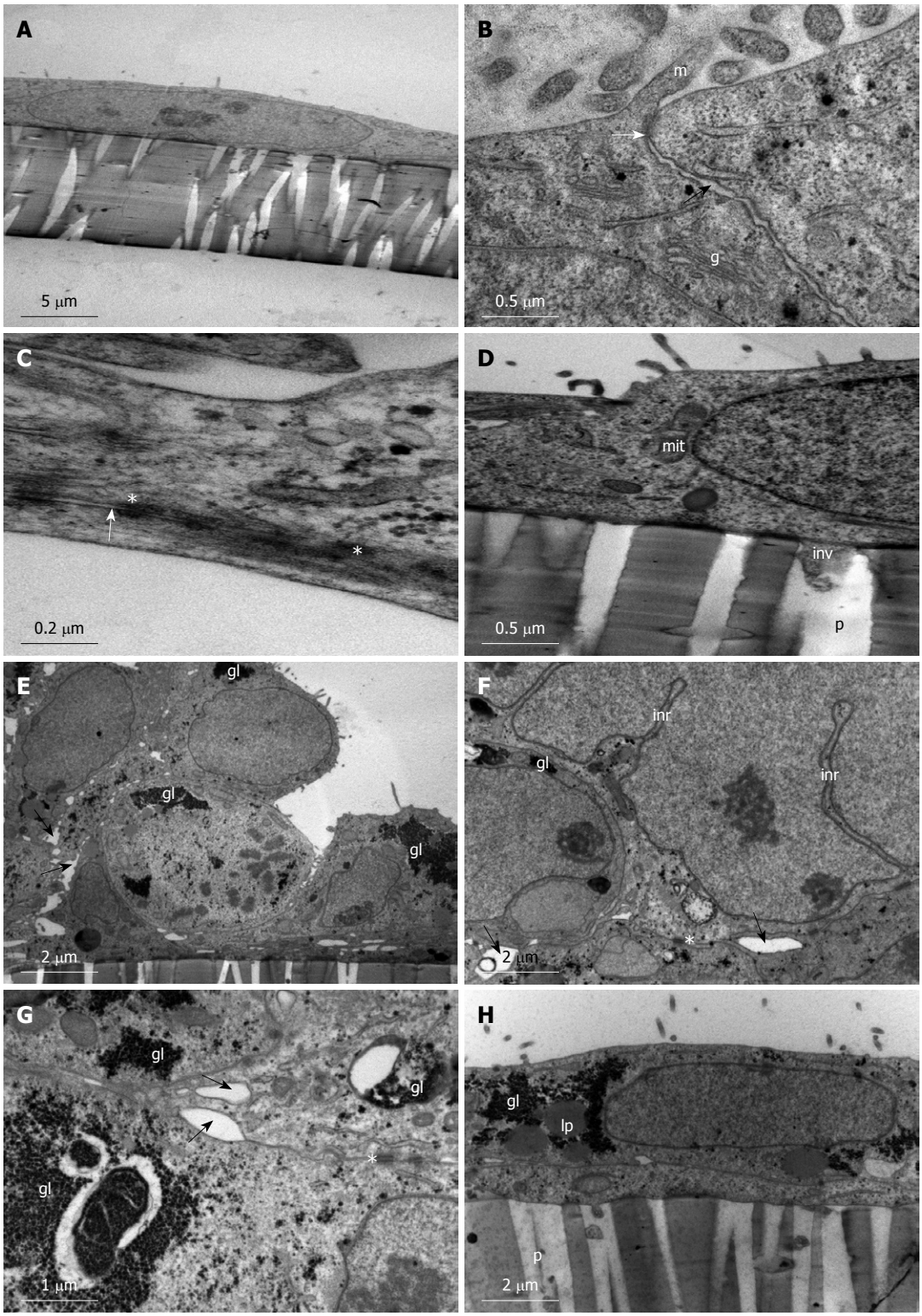


Figure 1 Spontaneous differentiation of Caco-2 into sub-population 1 of stratified cuboidal cells. A-D: Caco-2 cells grown on membrane filters examined on day 1 one post-confluency. A: Simple squamous cells with irregular sparse microvilli; B: Junction between two cells (black arrow) and establishment of a tight junction (white arrow) and the presence of microvilli (m) and golgi bodies (g); C: Desmosome (*) formation along the lateral plasma membrane between two cells (white arrow); D: Basolateral plasma membrane depicting attachment to membrane filter with evidence of small cell protrusions (inv) into the membrane pore (p). Mitochondria (mit); E-H: Caco-2 cells grown on membrane filters examined 12 d post-confluency. E: Cells appear stratified and cuboidal with large intercellular spaces (black arrows); large glycogen stores are evident (gl); F: Intercellular spaces (black arrows) and desmosomes (*) are evident between cells with glycogen deposits (gl) and indentations of the nucleus form large intranuclear rods (inr); G: High power image of intercellular spaces (black arrows) and desmosomes (*) present along the lateral plasma membrane with large glycogen deposits (gl) found in the cytoplasm; H: Basolateral plasma membrane showing attachment of the cell to the membrane filter. No invasive processes are evident projecting into the filter pores (p). Glycogen (gl) and some lipid (lp) droplets are present in the cell cytoplasm.

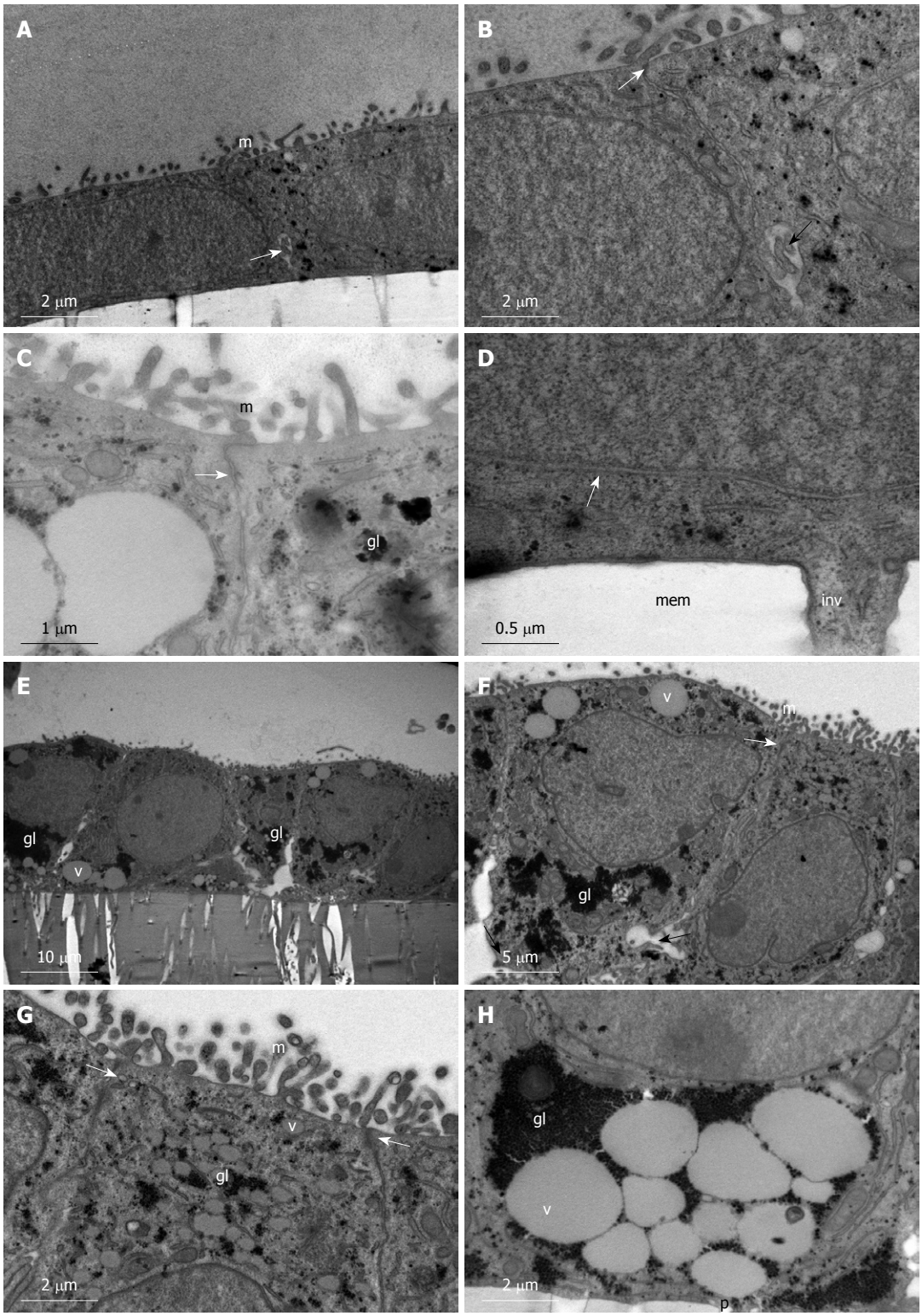


Figure 2 Spontaneous differentiation of Caco-2 into sub-population 2 of simple columnar monolayer of polarized epithelial cells. A-D: Caco-2 cells grown on membrane filters examined on day 1 post-confluency. A: Simple cuboidal epithelium with regular microvilli (m) and intercellular junctions (white arrow); B: Intercellular junction with a tight junction (white arrow) evident along the apical part of the lateral plasma membrane. A small intercellular space (black arrow) is evident near the basolateral region of the plasma membrane; C: High power image of a tight junction (white arrow) between cells showing glycogen particles (gl) and microvilli (m); D: Basolateral plasma membrane showing intercellular junction (white arrow) and small invasive projection (inv) into the filter pore. Membrane (mem); E-H: Caco-2 cells grown on membrane filters examined on day 12 post-confluency. E: Simple columnar polarized epithelium is present with large glycogen stores (gl), mucin filled vacuoles (v), and intercellular spaces along the basal part of the lateral plasma membrane; F: Junction between two columnar cells (white arrow); regular microvilli (m) are present as well as large glycogen stores (gl) and mucin filled vacuoles (v). Intercellular space (black arrow); G: High power micrograph showing the apical domain of cells with regular microvilli (m), glycogen stores (gl), mucin vacuoles (v), and tight junction formation (white arrows); H: High power image of the basal domain of cells with large mucin filled vacuoles (v) enclosed by large glycogen stores (gl). No invasive processes are evident projecting into filter pores (p).

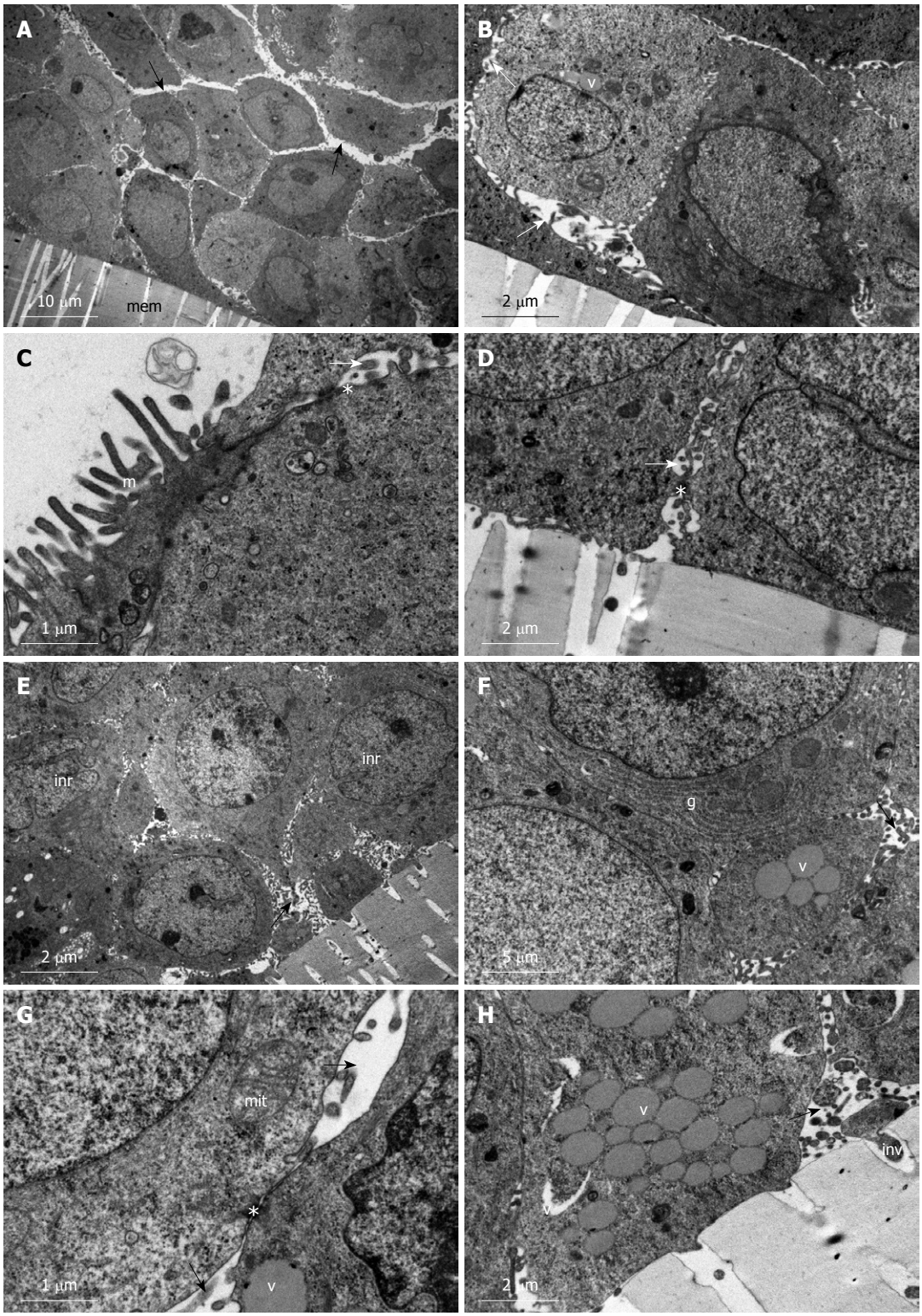


Figure 3 Undifferentiated heterogenous population of HT-29 cells. A-D: HT-29 cells grown on membrane filters (mem) and examined day 1 post-confluency. A: Stratified cuboidal cells arranged in clumps. Large intercellular spaces (black arrows) are evident with microvilli projecting into them; B: High power micrograph showing large intercellular spaces (approximate 2 μm) (white arrows) with microvillar projections and formation of small vacuoles (v); C: Microvillous (m) apical plasma membrane is present and desmosomes (*) are evident along contact sites of the lateral plasma membrane of adjoining cells and presence of intercellular spaces (white arrow); D: No invasive projections are evident along the basolateral plasma membrane. Desmosomes (*) are present at contact sites of the plasma membrane, which otherwise shows intercellular spaces (white arrow); E-H: HT-29 cells grown on membrane filters (mem) and examined day 12 post-confluency. E: Stratified cuboidal cells with microvillar projections entering the large intercellular space (black arrow). Intranuclear rods are evident (inr); F: High power micrograph of junction between two cells. Golgi bodies (g) as well as mucin filled vacuoles (v) are distributed throughout the cell cytoplasm and intercellular spaces are evident with microvilli projecting into their lumen; G: High power micrograph of a contact site between two opposing plasma membranes showing a desmosome (*). Large intercellular spaces (black arrows) are evident above and below the desmosomes contact site. Mitochondria (mit) and mucin filled vacuoles (v) are also evident; H: Basolateral attachment site showing minor invasive projections (inv) into the filter pore. Intercellular spaces (black arrow) and vacuoles (v) are evident.

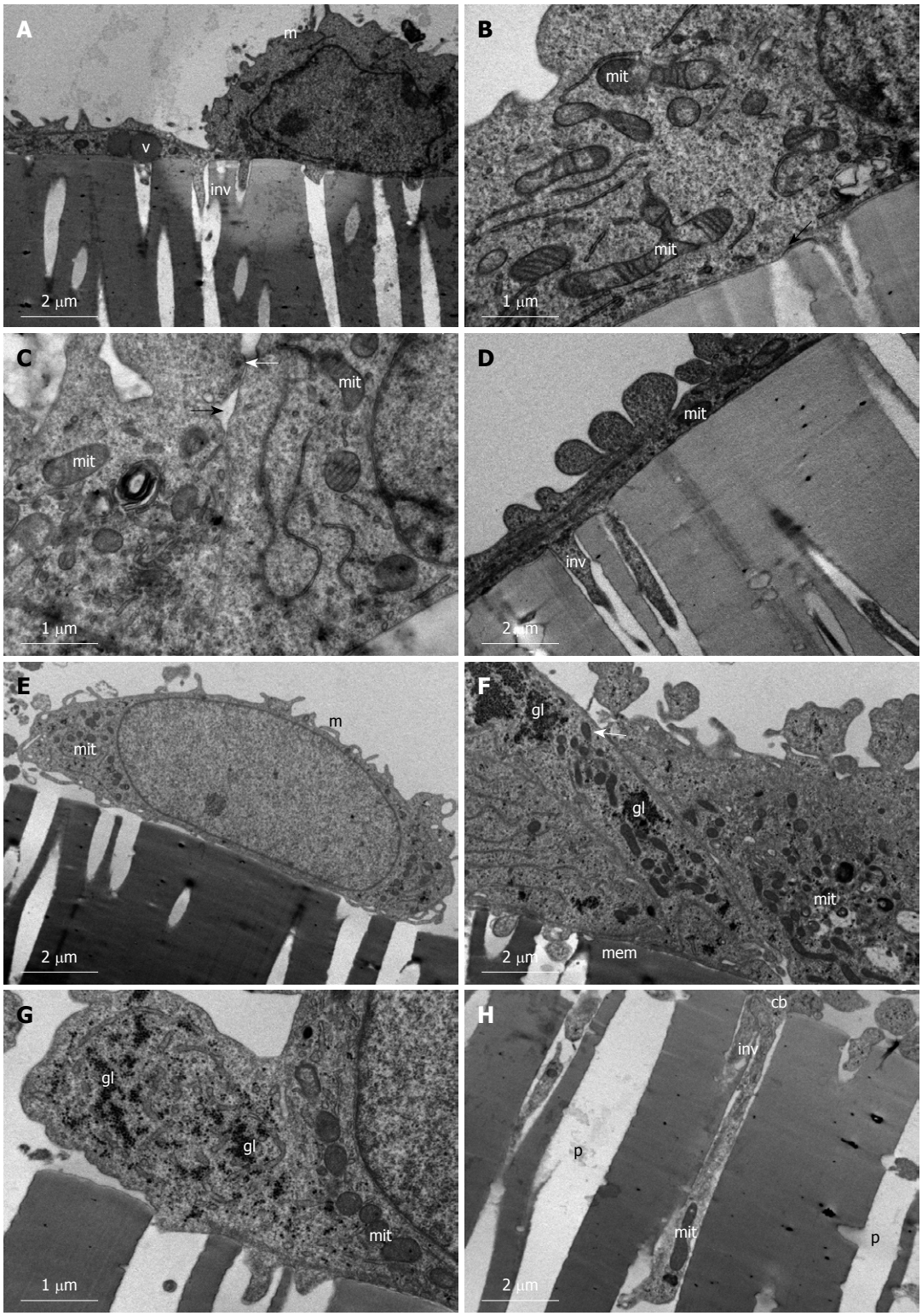


Figure 4 Undifferentiated heterogenous population of SW480 cells. A-D: SW480 cells grown on membrane filters and examined day 1 post-confluency. A: Simple squamous (left) and simple cuboidal (right) distinctions are already evident in cells on day 1 post-confluency. Cells exhibit vacuoles (v), microvilli (m), and invasive processes (inv) are evident projecting into filter pores; B: Large population of mitochondria (mit) are evident which exhibit branching characteristics; C: Intercellular junctions (black arrow) exhibit early formation of the tight junction (white arrow). Mitochondria (mit) with numerous cristae are present; D: Simple squamous cells exhibiting long invasive processes (inv) into the filter pores. Mitochondria (mit); E-H: SW480 cells grown on membrane filters and examined day 12 post-confluency. E: Cells remain a monolayer with microvillar (m) projections and abundant mitochondria (mit); F: Junction between two adjoining cells growing on membrane (mem) showing no intercellular spaces, tight junction formation (white arrow), abundant mitochondria (mit), and glycogen (gl) stores; G: High power micrograph depicting the glycogen (gl) granules in the cytoplasm in amongst mitochondria (mit); H: Basolateral region of cell body (cb) showing deep invasive processes (inv) projecting into filter pores (p). These processes extend to depths of approximately 10 μm and cellular organelles such as mitochondria (mit) are present in these processes, showing deep anchorage of cell.

enterocyte population when the original adenocarcinoma was excised. The polarisation of the Caco-2 epithelial layer indicated that epithelial organisation had been achieved[3] and the cells were contained in the epithelial monolayer without morphological indications of metastatic properties. The presence of glycogen however, suggested that neoplastic proliferation was still taking place in this population. This finding might be significant for tumor excision and treatment, as glycogen stores suggest a neoplastic cell; however, the morphological architecture indicates a contained tumor with possible clear margins and a better probability of complete surgical excision.

Sub-population 1 of Caco-2 cells did not form regular microvilli, and tight junctions did not polarise 12 d post-confluency. This cell population continued to grow in clumps of stratified cuboidal epithelium with large intercellular spaces between individual cells, suggesting high cellular motility (particularly in the apical population of cells)[24]. In the basal distribution of the cells, desmosomes were evident in areas of established cellular contact. Glycogen particles were abundant, with both an apical and basal distribution. Another additional feature that became apparent using a combined osmium tetroxide and potassium ferrocyanide selective staining, which would otherwise not have been adequately sampled, was the presence of intranuclear rods. These structures are indentations of the cytoplasm into the nucleus, whereby they increase the nucleocytoplasmic interactions[36] and carry out nucleocytoplasmic exchange, which is the most important transport phenomena in cells[37]. In the case of malignant colorectal cancer cells, intranuclear rods are suggested to carry out nucleocytoplasmic exchange of glycogen[37]. In a previous report in the Ehrlich-Lettré mouse ascites tumor, glycogen production that originated in the nucleus shifted from the nucleus to the cytoplasm *via* a transfer mechanism that allows the intranuclear glycogen to pour out into the cytoplasm *via* the intranuclear indentations (rods)[37]. Intranuclear rods are prevalent in the Caco-2 cell line (in particular sub-population 1); however, glycogen is predominantly expressed in the cytoplasm, which suggested that the nucleocytoplasmic exchange has occurred. Due to their disorganized arrangement, large intercellular spaces and lack of polarisation and tight junctional formation, sub-population 1 of Caco-2 cells did not form an impermeable monolayer or "classical epithelium". A lack of tight junction formation allows for dissociation of individual cells from the primary tumor due to a decrease in cell-cell adhesion, which consequently allows for the cancerous cells to invade the surrounding tissue, leading to metastasis[38]. Furthermore, for successive metastasis to occur, the tight junctions present in the surrounding tissue must be compromised to enable the tumor cells to penetrate[38]. In a previous investigation of colorectal cancer cells, using the tight junctional protein claudin-4 as a marker, a disruption of claudin-4 mediated tight junction formation enhanced cancer cell invasion and metastasis in colorectal cancer[3]. Finally, the morphology of the cells in sub-population 1 of the Caco-2 cell line did not provide strong evidence indicating the formation of enterocytes, and the high glycogen content signifies metastatic proliferation. Thus, data associated with future functional and transport studies carried out on sub-population 1 of Caco-2 cells might be restricted.

When grown under standard conditions, the HT-29 cells, although not homogenous, did not differentiate into a monolayer of polarized cells, as there is no evidence of any tight junction formation, which again suggests that cells can break away from the primary tumor and begin a metastatic process in surrounding tissue. These results are consistent with previous reports of a heterogenous HT-29 cell population[31,33,39]. At day 1 post-confluency, the cells were arranged into a large clump of microvillous stratified cuboidal epithelium up to seven layers thick. By day 12 post-confluency, large intercellular spaces were observed suggesting that cells remain relatively motile[24], even though some desmosomes were evident at points of plasma membrane contact of cells in the basal population. No invasive cellular projections were evident in the membrane filters, and trace amounts of glycogen particles were present in HT-29 cells when compared to the abundant glycogen stores found in both populations of Caco-2 cells. Reduced glycogen storage suggested that these cells were not undergoing comparable metastatic proliferation as the two sub-populations of the Caco-2 cell line. Previous glycogen assay studies have provided comparative data, where less glycogen content was present in HT-29 cells during both the exponential and stationary growth phase than in Caco-2 cells[10], indicating that the selective staining produced by the potassium-ferrocyanide fixation as a glycogen marker yields comparative results. An additional feature of the HT-29 cell line was the presence of mucin filled vacuoles, which provides evidence that these cells potentially originated from a mucous secreting population of the intestinal epithelium. Previous investigations of the HT-29 cell line have also isolated mucous-secreting sub-clones in the population, but this was achieved by inducing cellular differentiation by the administration of galactose to the culture medium[31]. This induced differentiation also resulted in polarisation and monolayer formation of the HT-29 cells[31]. The present study did not include differentiation of HT-29 cells by adding galactose; therefore, the presence of the mucin filled vacuoles might be indicative of a population of precursor cells that later form secretory intestinal epithelial cells.

When the SW480 cells were cultured under standard conditions, a non-homogenous population of undifferentiated cells was observed. One cell type was simple squamous with irregular microvilli, and the other population of cells were simple spindle-shaped with irregular microvilli. These results were similar to those reported by previous investigations, which also described a heterogenous undifferentiated population of SW480 cells with comparable morphological features[35]. By day one post-confluency, cell-to-cell contacts have been established, with no evidence of intercellular spaces; however, no desmosomes or tight junctions were evident. The glycogen content was equivalent in both populations of SW480 cells, indicating that comparable metastatic properties were present in both sub-populations of this cell line. By day one post-confluency, abundant mitochondria were

present throughout the cell cytoplasm when compared to the cytoplasm of the other two cell lines, and some mitochondria appeared to be branching. Large numbers of mitochondria indicated that these cells were highly active.

At 12 d post-confluency, glycogen particles were also evident, representing intermediate glycogen expression when compared to the large amounts found in Caco-2 cells and trace amounts found in the HT-29 cells. The glycogen stores were distributed amongst mitochondrial populations; however, no intranuclear rods were evident projecting into the nucleus. Another striking feature of the SW480 cell line, irrespective of cell type, was their ability to send deep invasive processes into the membrane filter pores (approximate 8 μm deep). These processes often contained cell organelles, such as mitochondria, which demonstrated deep cell anchorage. Previous reports have also reported that spindle-shaped SW480 cells exhibited vast migration through uncoated membrane filters, whereas cells with a more epithelioid shape (Caco-2 and HT-29) scarcely invaded membranes, even those coated with Matrigel[6]. These potentially invasive characteristics were not observed in Caco-2 or HT-29 cells in this investigation. Invasive processes have, however, been reported in Caco-2 cells in other literature when invasion was induced by adding a collagen matrix to the membrane filters[19]. The addition of the collagen promoted cell migration through the filter pores, which eventually led to the depolarisation of the model cells[19]. No invasion inducing agents were administered in this investigation and there is clear evidence that the SW480 cells presented the most invasive capabilities of the three cell lines. These results were comparable with a previous investigation where metastatic capacities were examined in the SW480 cell line using orthotopic tumor models[40]. When SW480 cancer cells were injected or implanted into the caecum of mice, SW480 cells had a 100% take rate, when compared to a take rate of 69% in HT-29 and 40% in Caco-2 cells. The SW480 cells also had the highest growth index, which was represented by the time it took between implantation and the appearance of disease symptoms[40]. The ultrastructural features observed in this study of the SW480 cancer cell line supported other literature that considered this cell line to be highly malignant[35].

In conclusion, this was the first study to incorporate selective staining for transmission electron microscopy to help identify glycogen storage and nucleocytoplasmic exchange of glycogen by identifying intranuclear rods. Ultrastructurally, the loss of the tight junction in sub-population 1 of Caco-2 cells, and in HT-29 and SW480 cells is indicative of highly disorganized epithelia, whereby individual tumor cells have the potential to dissociate from the primary tumor and begin a metastatic process in other tissue. Conversely, sub-population 2 of Caco-2 cells remained as a polarized monolayer of epithelia with extensive tight junction formation, suggesting organised epithelia with strong cell-cell adhesion with lower disassociation potential. Their glycogen storage, however, suggested that Caco-2 and SW480 cells were undergoing the largest amount of neoplastic transformation, and the abundant mitochondria present in both populations of SW480 cells was also indicative of that feature. The ultrastructural features examined in this study have contributed to our already vast knowledge of colorectal cancer cells. We propose the structural parameters detailed in this systemic study can be used as a quality control in the study of the ultrastructure of Caco-2, HT-29, and SW480 cells *in vitro*. The table herein presented might serve as a morphometric guide for others who wish to utilise these cells lines. Future 3D tomographical work will further capture the nucleocytoplasmic exchange and assist in identifying other key ultrastructural regulators of the invasive process of malignant cells. The identification of glycogen stores in these cells might result in drug therapy trials targeted against these abundant intracellular features.

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COMMENTS

Background

The extensive variability that exists in the morphology, growth rate, and cell viability between different colorectal cancer cells in culture results in wide-ranging observations and the inability to standardise data. This investigation is designed to compile relevant ultrastructural data on three commonly used cancer cell lines to determine whether ultrastructural features and the presence of large glycogen stores can be used as indicators for the metastatic properties of cells.

Research frontiers

Large quantities of glycogen stores have been reported in a variety of cultured cells, particularly cells undergoing neoplastic transformations, even when the cells from the tissue of origin lack glycogen. By incorporating selective staining using an osmium tetroxide and a potassium ferrocyanide solution in routine transmission electron microscopy, these glycogen stores can be ultrastructurally identified and used as diagnostic markers for the metastatic properties of cells.

Innovations and breakthroughs

This is the first study to combined ultrastructural and functional features of colorectal cancer cells with selective staining for other neoplastic markers, such as glycogen.

Applications

The compilation of ultrastructural features and glycogen storage data from three commonly used colorectal cancer cell lines will assist future investigators in selecting appropriate cell models depending on their area of interest, particularly for drug therapy trials that might be directed against excessive intracellular glycogen stores.

Peer review

This investigation emphasizes the variability in morphology, behaviour, and metastatic properties of colorectal cancer cells and provides readers with a coherent guide on three commonly used colorectal cancer cell lines for use as a morphometric tool for future investigations.

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ORIGINAL ARTICLE

Hydrophobic protein in colorectal cancer in relation to tumor stages and grades

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Abstract

AIM: To identify differentially expressed hydrophobic proteins in colorectal cancer.

METHODS: Eighteen pairs of colorectal cancerous tissues in addition to tissues from normal mucosa were analysed. Hydrophobic proteins were extracted from the tissues, separated using 2-D gel electrophoresis and analysed using Liquid Chromatography Tandem Mass Spectrometry (LC/MS/MS). Statistical analysis of the proteins was carried out in order to determine the significance of each protein to colorectal cancer (CRC) and also their relation to CRC stages, grades and patients' gender.

RESULTS: Thirteen differentially expressed proteins which were expressed abundantly in either cancerous or normal tissues were identified. A number of these proteins were found to relate strongly with a particular stage or grade of CRC. In addition, the association of these proteins with patient gender also appeared to be significant.

CONCLUSION: Stomatin-like protein 2 was found to be a promising biomarker for CRC, especially in female patients. The differentially expressed proteins identified were associated with CRC and may act as drug target candidates.

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Key words: Biomarker; Colorectal cancer; Liquid chromatography tandem mass spectrometry; Proteomic approach; 2D-polyacrylamide gel electrophoresis

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INTRODUCTION

Cancer of the colon and rectum is termed colorectal cancer (CRC). CRC is ranked the third most common cancer worldwide. Statistical data from the World Cancer Report revealed that there are more than 940 000 cases of CRC each year with an annual mortality of around 500 000[1]. In Malaysia, CRC is the most common type of cancer amongst men and the 3rd most common cancer amongst women after breast and cervical cancer[2].

Proteomics in cancer is used to study the protein expression pattern of normal and cancerous tissues in order to elucidate the molecular mechanism associated with disease development and progression[3]. Membrane proteins which make up approximately 30% of the human proteome are important components of cells[4]. Membrane-associated proteins are involved in various

fundamental biological processes in cells including signal transduction, immune regulation and transportation[5]. Nevertheless, the hydrophobic nature of membrane proteins make the extraction of these proteins challenging as they are difficult to extract and analyse[6]. Solubilisation of membrane proteins requires the use of stringent reagents and the addition of urea and thiourea[7,8]. Moreover, these proteins are prone to precipitation at their isoelectric points which may lead to protein loss during 2D-Polyacrylamide Gel electrophoresis (2D-PAGE) separation[9,10]. Thus, the proteome of membrane proteins is normally under-represented. Many investigations have been carried out to improve extraction, enrichment and separation of membrane and membrane-associated proteins[11,12].

In this study, a mixture of reagents was added to the extraction buffer in order to increase the solubility of membrane proteins. Although it is impossible to extract all the membrane proteins from tissues, we obtained a consistent pattern of protein maps for cancerous and normal tissues, respectively. This enabled us to identify unique or differentially expressed membrane proteins in CRC tissues which may be useful for the diagnosis or treatment of CRC. The significance of these proteins in relation to cancer stages, grades and patient gender was also evaluated.

MATERIALS AND METHODS

Tissue samples

Surgically removed colorectal cancerous tissues and their respective normal mucosa were obtained from 18 patients who received treatment at Penang General Hospital, Malaysia. Written informed consent was obtained from all patients before surgery. The tissue specimens were kept in -80℃ until analysis. The tissues were grouped according to cancer stage, grade and patient gender (Table 1). The tumor type obtained was adenocarcinoma and normal mucosa was obtained from a site at least 10 cm away from the tumor. None of the patients received preoperative neo-adjuvant chemotherapy or radiotherapy. All the patients were > 40 years old at the time of surgery. The tissues were pathologically confirmed by the hospital's pathologist. Frozen sections of tissue were taken from cancerous tissues in the anterior and deep region to ensure adequacy of tumor and only cancerous tissue that contained > 90% malignant cells was used in this study.

Protein extraction

The membrane or membrane-associated proteins were extracted from the homogenised tissues using thiourea buffer (7 mol/L urea, 2 mol/L thiourea, 4% CHAPS and 0.2% carrier ampholytes). Briefly, 200 mg of tissue was homogenised in 1 mL of Tris buffer (40 mmol/L Tris) followed by sonication for 30 s and chilled on ice for 2 min. The lysate was then vortexed for 5 min before being centrifuged (12000 r/min, 15 min, 20℃). The supernatant was kept for a separate experiment while the pellet was rinsed twice with Tris buffer. The pellet was then suspended in 150 μL of thiourea buffer, sonicated, vortexed and centrifuged (12000 r/min, 15 min, 20℃) and the supernatant was collected for analysis.

Table 1 Clinical information of the patients studied

No.	Age (yr)	Sex	Duke's system	Localization	Degree of differentiation
1	57	F	C	Right colon	Well-differentiated
2	64	M	C	Caecum	Moderately-differentiated
3	45	M	B	Sigmoid colon	Well-differentiated
4	91	F	C	Sigmoid colon	Well-differentiated
7	75	F	B	Rectosigmoid	Well-differentiated
8	62	M	C	Rectum	Well-differentiated
9	71	F	C	Sigmoid colon	Moderately-differentiated
10	76	M	B	Rectosigmoid	Well-differentiated
12	70	M	C	Sigmoid colon	Moderately-differentiated
13	63	M	B	Rectum	Moderately-differentiated
14	62	F	B	Rectosigmoid	Well-differentiated
15	54	F	B	Descending colon	Well-differentiated
17	74	M	B	Rectosigmoid	Moderately-differentiated
19	67	M	B	Rectum	Moderately-differentiated
20	53	F	C	Rectosigmoid	Moderately-differentiated
21	79	F	C	Rectosigmoid	Moderately-differentiated
23	67	M	C	Sigmoid colon	Moderately-differentiated
24	42	M	B	Descending colon	Well-differentiated

2-D gel electrophoresis

The protein concentration of the thiourea buffer extracts was determined using the RCDC protein assay kit (Bio-Rad, USA), and 500 μg of the extract in 185 μL of rehydration buffer (same composition as thiourea buffer) was used to rehydrate IPG strips (4-7 pH, 11 cm) for 15 h and focused using IEF Cell (Bio-Rad, USA) starting from 0 to 250 V within 15 min, followed by 250 to 8000 V within 2.5 h and maintained at 8000 V until 35000 V-h was achieved. Subsequently, the IPG strips were equilibrated for 15 min with gentle shaking in Sodium Dodecyl Sulfate-Polyacrylamide Gel electrophoresis (SDS-PAGE) Equilibration Buffer I (6 mol/L urea, 0.375 mol/L Tris pH 8.8, 2% SDS, 20% glycerol, 2% DTT and a trace amount of bromophenol blue), followed by another 15 min of gentle shaking in SDS-PAGE Equilibration Buffer Ⅱ (same composition as SDS-PAGE Equilibration Buffer 1, however, 2.5% iodoacetamide was used instead of 2% DTT). Second dimension separation was carried out under constant voltage of 200 V for approximately 3 h in 10% SDS-PAGE. The gel was stained overnight using Coomassie Blue (0.2% Coomassie Brilliant Blue R250, 50% MeOH and 2% acetic acid) and destained for 2 h.

Gel imaging and statistical analysis

The 2D-PAGE images were acquired by using the Versa-doc system (Bio-Rad, USA). The gel images were processed and analyzed using PDQuest version 7.3 (Bio-Rad, USA). The software was used to create a matchset to compare the images of cancerous and normal colorectal tissues. The matchset was used to analyze quantitative

and qualitative differences in protein spots between the images. The intensity of the protein spot was measured after normalization of the protein spot as a percentage of the total density of all proteins spots on each gel in order to minimise the variation which may be caused by the amount of sample loaded. β-actin protein spot was used as the landmark for gel image analysis. A protein was up-regulated if its expression level in cancerous tissues was 1.5-fold or more compared to normal colorectal tissue, and was down-regulated when the opposite occurred. A protein was uniquely expressed if it was found exclusively in either normal or cancerous colorectal tissue only. In addition, the statistical significance of the protein's change in expression was determined using the Wilcoxon signed-rank test (PDQuest version 7.3) at 95% level of confidence.

In-gel digestion

In-gel digestion was carried out according to Gam *et al*[13]. In brief, the protein spots of interest were excised from the gel. The Coomassie blue stain on the protein spot was removed by dehydrating the gel pieces in acetonitrile and rehydrating in 100 mmol/L NH_4HCO_3. This step was repeated three times. The gel pieces were then incubated in a volume of trypsin buffer (50 mmol/L NH_4HCO_3 and 5 mmol/L $CaCl_2$) containing 12.5 ng/μL trypsin and chilled at 4℃ for 45 min. The trypsin buffer with trypsin was then replaced by trypsin buffer without trypsin at a volume sufficient to wet the gel pieces and incubated overnight at 37℃. The peptides were then eluted from the gel pieces and dried using a sample dryer (Techne, UK) under a continuous flow of nitrogen gas and stored at -20℃ prior to analysis.

Liquid chromatography tandem mass spectrometry analysis

The dry peptides were suspended in 15 μL of ultrapure ddH_2O and were subjected to liquid chromatography tandem mass spectrometry (LC/MS/MS) analysis using an electrospray ionisation-ion trap mass spectrometer (Agilent). A 5 μL volume of the reconstituted sample was injected into a RPC-column (C18, 300 Å, 5 μm, 1 mm × 150 mm) connected to a HPLC (1100 Series, Agilent). A capillary pump was used to pump the mobile phase (A and B) at a flow rate of 15 μL/min. The linear gradient used was 5% B to 95% B in 65 min. Mobile phase A was 0.05% formic acid in deionized water and B was 0.05% formic acid in acetonitrile. The HPLC was interfaced to the mass spectrometer detector. An experimental method comprising 2 scan events was used for analysis. The first scan event was a full scan MS whilst the second scan was the data dependent MS/MS scan which is dependent on the results of the first scan event. Two of the most intense ions in the MS scan which surpassed the threshold set were automatically isolated and excited to the MS/MS scan. The MS parameters used were; dry gas flow rate of 15 μL/min, nebulizer pressure of 30.0 psi and dry gas temperature of 300℃. The parameters for the MS/MS scan were; default collision energy (voltage) of 1.15 V, charge state of 2, minimum threshold of 1000 counts, and isolation width of 2 m/z. The MS/MS data from the analysis were used to search for their corresponding protein identity in Swiss-Prot using MASCOT Search engine version 2.2 from Matrix Science (www.matrixscience.com). The search parameters used were *Homo sapiens* for taxonomy, trypsin for enzyme, carboxymethyl for fixed modifications, peptide tolerance of +/- 2 Da, MS/MS tolerance of +/- 0.8 Da and average experimental mass value. Further analysis of proteins was carried out using the ProtParam programme available at the EXPASY website (http://www.expasy.org/tools/protparam.html) for calculation of the proteins grand average of hydrophobicity (GRAVY). The Tmpred programme (http://www.ch.embnet.org/software/TMPRED_form.html) was used to determine the transmembrane domain of the proteins.

Western blotting

Western blotting was carried out using a semi-dry blotting method[14]. Protein extracts were separated by SDS-PAGE according to Laemmli[15]. A similar quantity of protein was loaded on to SDS-PAGE, after electrophoresis separation, the proteins in the gel were transferred using a TE 70 Semiphor semi-dry transfer unit (Hoefer Scientific, Germany) at 134 mA for 1.5 h to a nitrocellulose membrane. The membrane was incubated in 20 mL of mouse anti-SLP-2 antibody (Abnova, Taiwan) at 1:250 followed by incubation with 50 mL of HRP conjugated anti-mouse secondary antibody (Bio-Rad, USA). The reaction of HRP and its substrate 4-Chloro Naphthol (4CN) indicated the presence of stomatin-like protein 2 (SLP-2).

RESULTS

Two-dimensional gel electrophoresis for protein separation was carried out on a linear pH range of 4 to 7. A total of 13 differentially expressed proteins which were expressed abundantly in either cancerous or normal tissues were identified (Figure 1). Identification of differential protein expression in individual patients was accomplished by conducting a pair-wise comparison between the cancerous and normal tissues for each patient and is displayed in Figure 2. An average of 177.35 ± 26.60 protein spots was detected on 2D gel, with a coefficient variation of 15%. Eight proteins, namely tubulin α-1 chain (S4), tubulin β-2 chain (S5), chaperonin GroEL (S6), heat shock 70 kDa protein (S7), SLP-2 (S8), annexin A3 (S9), annexin A4 (S10) and ATP synthase D chain (S13) were up-regulated although only the up-regulation of tubulin β-2 chain, SLP-2, annexin A3 and annexin A4 were significant ($P < 0.05$) in CRC. Figure 3 shows the comparative analysis of spot intensity between normal and cancerous tissues for SLP-2, annexin A3 and annexin A4.

The identity of the proteins was determined by amino acid sequencing *via* tandem mass spectrometric analysis followed by protein database search. The representative

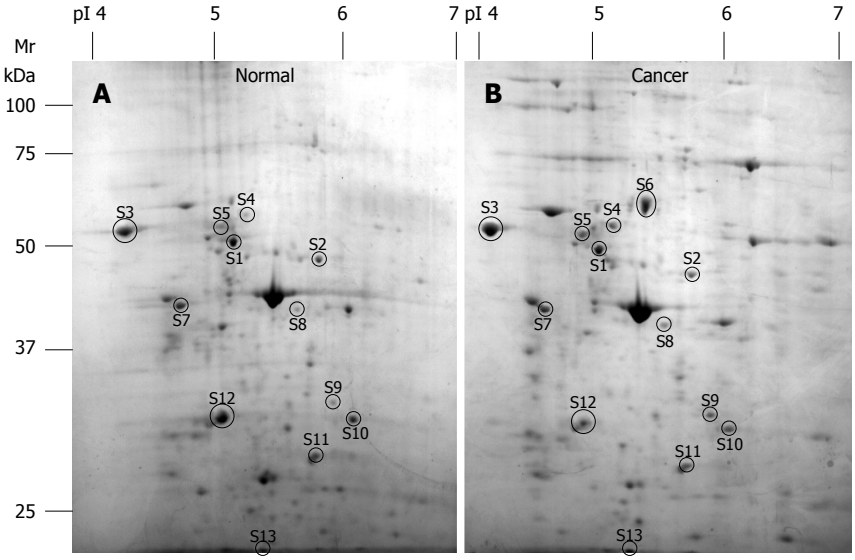


Figure 1 Protein expression profiles of normal mucosa (A) and CRC tissues (B). S1: F1-ATPase β subunit; S2: Ubiquinol-cytochrome C reductase; S3: Calreticulin; S4: Tubulin α-1 chain; S5: Tubulin β-2 chain; S6: Chaperonin GroEL; S7: Heat shock 70 kDa protein 5; S8: Stomatin-like protein 2; S9: Annexin A3; S10: Annexin A4; S11: Prohibitin; S12: Annexin V; S13: ATP synthase D chain.

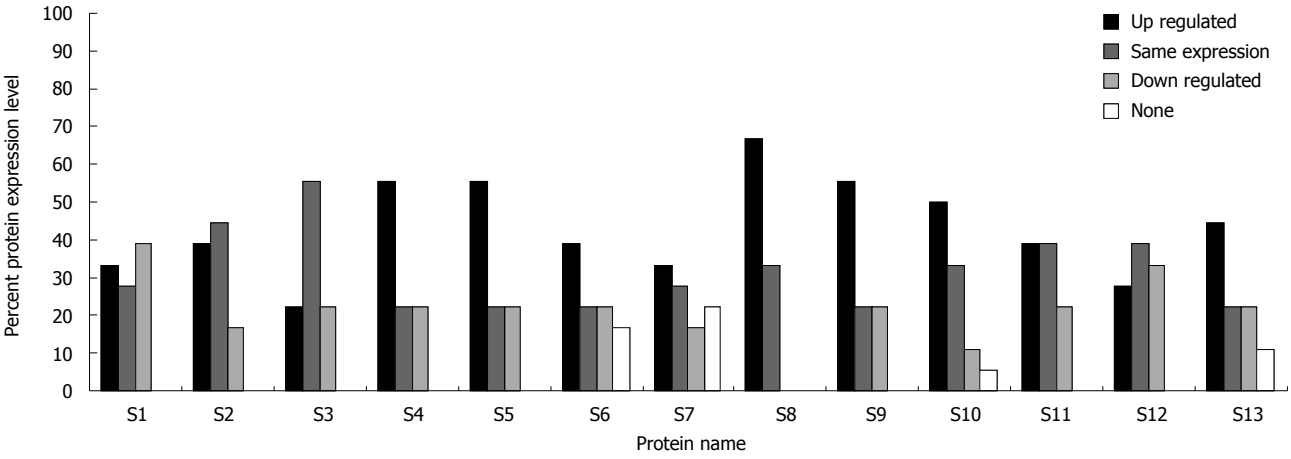


Figure 2 Distribution of proteins in cancerous tissues *vs* normal tissues in all 18 patients. An up-regulated protein is a protein with a higher expression level in cancerous tissues than in normal tissues, and *vice versa* for down-regulated proteins. Same expression is when the expression levels are the same between cancerous and normal tissues. None expression is when the protein was not found in either cancerous or normal tissues.

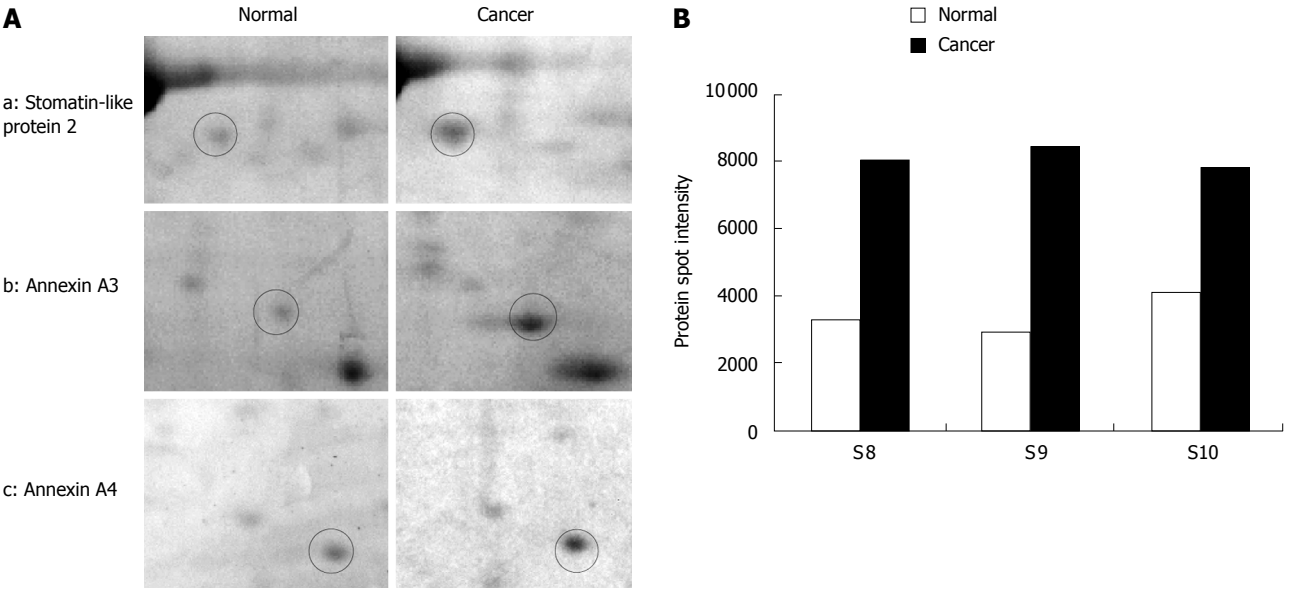


Figure 3 Protein spot analysis (A) and quantification of spot intensity (B) of (a) stomatin-like protein 2 (S8), (b) annexin A3 (S9) and (c) annexin A4 (S10) from normal and colorectal cancerous tissues in the same patient.

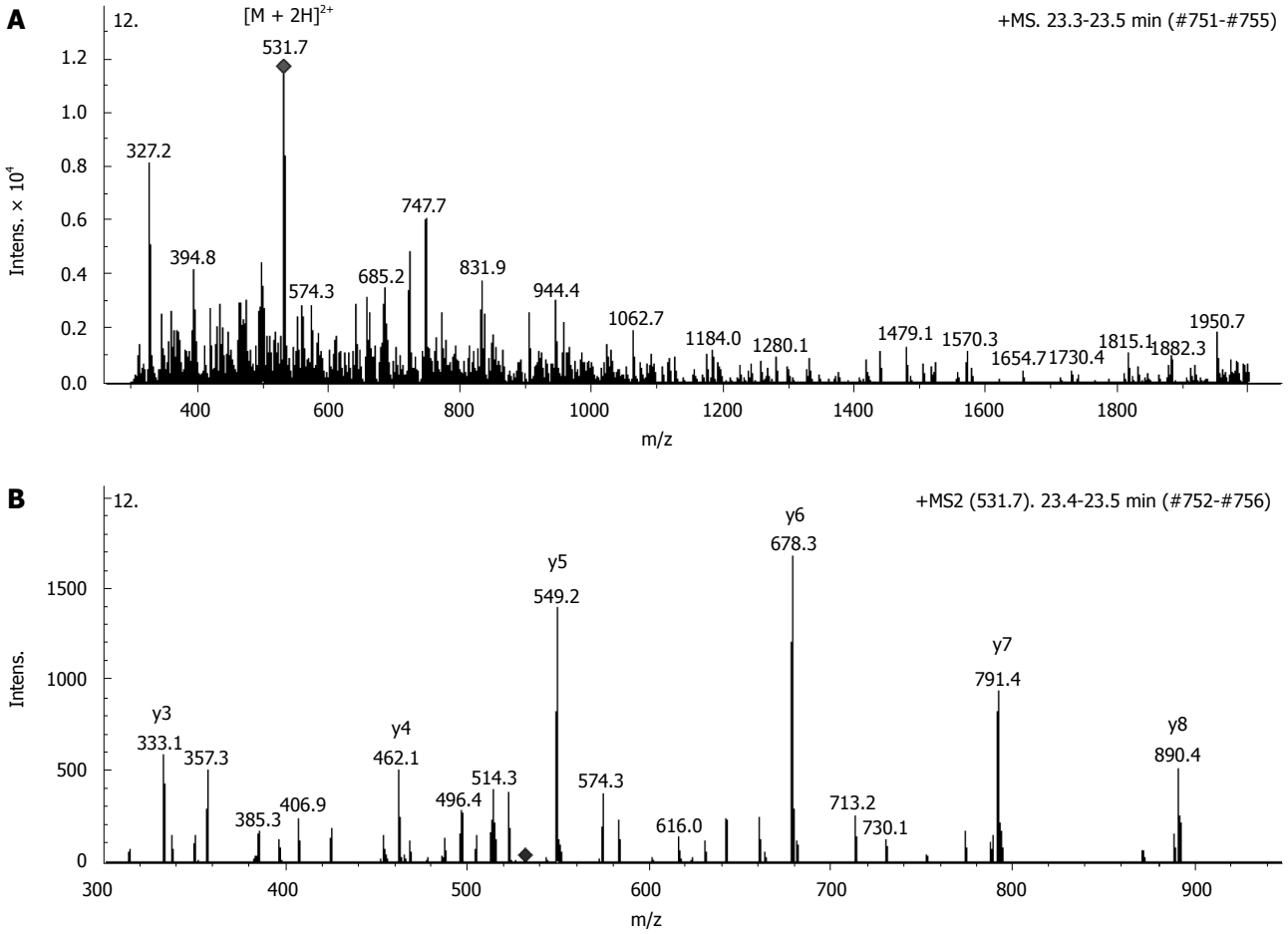


Figure 4 LC/MS/MS analysis of protein. A: Full scan MS spectra of 531.7 m/z peptide; B: MS/MS spectra of 531.7 m/z peptide ions of stomatin-like protein 2.



Figure 5 Western blotting for stomatin-like protein 2. Lane 1 and 2 are proteins extracted from normal and cancerous tissues, respectively, from the same patient.

MS and MS/MS spectra of SLP-2 are shown in Figure 4. Western blotting was used to confirm the results obtained from mass spectrometric analysis. Figure 5 shows a Western blot image of SLP-2 extracted from normal and cancerous tissue in the same patients, SLP-2 was only detected in cancerous tissue. Table 2 shows the 13 differentially expressed proteins identified in this study. The GRAVY score indicated the hydrophobic property of each protein, and the change in the protein expression levels is indicated as fold change (calculated as the ratio of total spot intensity of the protein in normal and cancerous tissues in all 18 patients). A positive value indicated that the protein expression level was higher in cancer compared to normal tissue or that it was up-regulated, while a negative value showed that the protein was down-regulated. Chaperonin GroEL was shown to have the greatest fold change (+265.0) although its up-regulation in all 18 patients was not statistically significant ($P < 0.05$).

Analysis of protein expression was carried out by comparing protein expression profiles of cancerous and normal tissues within and between cancer stage, pathological status (grade) and gender of the patients. The tissue specimens collected comprised 9 each of Duke's B and Duke's C, respectively. Figure 6A shows the level of up-regulation of the 13 proteins in Duke's B and C. The proteins which were up-regulated in > 50% of Duke's B cancer were SLP-2 (S8) and annexin A4 (S10), while F1-ATPase β subunit (S1), uniquinol-cytochrome c reductase (S2), tubulin α-1 chain (S4), tubulin β-2 chain (S5), SLP-2 (S8), annexin A3 (S9) and ATP synthase D chain (S13) were up-regulated in > 50% of Duke's C cancer. The up-regulation of both SLP-2 and annexin A4 was significant ($P < 0.05$) in Duke's B cancer, whilst only tubulin β-2 chain and SLP-2 were significantly ($P < 0.05$) up regulated in Duke's C cancer.

As for cancer grade, the tissues collected comprised 9 each of well-differentiated adenocarcinoma (WDC) and moderately-differentiated adenocarcinoma (MDC), respectively. Figure 6B shows the level of up-regulation of the 13 proteins in WDC and MDC. SLP-2 was expressed at > 50% in WDC and its up-regulation in WDC was

Table 2 Differentially expressed proteins in CRC

Protein spot	Protein name	SwissProt accession No.	Score	Theoretical molecular weight (Da)	Theoretical pI	Sequence coverage (%)	GRAVY	Fold change[1]	TMR
S1	F1-ATPase β subunit	P06576	364	58013	5.80	19	-0.030	+58.5	1
S2	Ubiquinol-cytochrome c reductase	P31930	171	53308	5.94	7	-0.141	+32.8	1
S3	Calreticulin	P27797	73	47092	4.30	11	-1.191	-31.9	1
S4[1,f]	Tubulin α-1 chain	Q71U36	61	50800	4.94	6	-0.229	+10.8	1
S5[1,c,f]	Tubulin β-2 chain	P68371	299	48142	4.70	25	-0.347	+20.8	1
S6	Chaperonin GroEL	P10809	185	61348	5.70	8	-0.076	+265.0	3
S7	Heat shock 70 kDa protein	P11021	775	72488	5.07	42	-0.487	+5.1	1
S8[a,b,c,d,e,f,g]	Stomatin-like protein 2	Q9UJZ1	151	38644	6.88	28	-0.161	+27.9	1
S9[a,e]	Annexin A3	P12429	140	36396	5.63	22	-0.430	+34.9	0
S10[a,b,c,e,f]	Annexin A4	P09525	165	35983	5.85	33	-0.447	+29.6	0
S11	Prohibitin	P35232	421	29890	5.57	41	+0.024	+11.8	1
S12	Annexin A5	P08758	195	35994	4.94	39	-0.330	-5.2	0
S13	ATP Synthase D chain	O75947	117	18406	5.22	32	-0.569	+8.3	0

[1]Fold change is given as the ratio of the spot intensity in normal mucosa over tumour tissue (negative variation or decrease) or tumor tissue over normal tissue (positive variation or increase). [a]Differentially expressed proteins that are statistically significant ($P < 0.05$) among all 18 patients; [b]Differentially expressed proteins that are statistically significant ($P < 0.05$) in Duke's B stage; [c]Differentially expressed proteins that are statistically significant ($P < 0.05$) in Duke's C stage; [d]Differentially expressed proteins that are statistically significant ($P < 0.05$) in WDC; [e]Differentially expressed proteins that are statistically significant ($P < 0.05$) in MDC; [f]Differentially expressed proteins that are statistically significant ($P < 0.05$) in female patients; [g]Differentially expressed proteins that are statistically significant ($P < 0.05$) in male patients. pI: Isoelectric point; GRAVY: Grand average of hydrophobicity; TMR: Transmembrane region.

significant ($P < 0.05$). Tubulin α-1 chain (S4), tubulin β-2 chain (S5), SLP-2 (S8), annexin A3 (S9) and annexin A4 (S10) were up-regulated in > 50% MDC, however, only the expression levels of SLP-2, annexin A3 and annexin A4 were significant ($P < 0.05$).

There were 10 male patients and 8 female patients included in this study. All 13 proteins identified in the study were equal to or > 50% up-regulated in female patients except for calreticulin (S3) and heat shock 70 kDa protein (S7) (Figure 6C). In contrast, all 13 proteins were up-regulated in < 50% of male patients except SLP- 2 (S8) and annexin A3 (S9) which were up-regulated in 50% of male patients (Figure 6C). Only SLP-2 was significantly up-regulated ($P < 0.05$) in CRC male patients, while tubulin α-1 chain, tubulin β-2 chain, SLP- 2 and annexin A4 were significantly up-regulated in CRC female patients.

DISCUSSION

The potential use of membrane proteins in drug targeted therapy and diagnosis of diseases is enormous, where many of the biomarkers for indication of diseases are of membrane origin[16]. The membrane or membrane-associated proteins contain potential antibody recognition sites that may provide identification of cancer development[6]. Although the extraction of membrane proteins is difficult, a combination of reagents can be used to enhance the solubility of membrane proteins. In this study, tissues were homogenised and treated with Tris buffer prior to thiourea buffer extraction. This pre-treatment removed aqueous soluble proteins and minimised proteolysis. Thus, the remaining tissue pellet contained mainly hydrophobic proteins which were then extracted using thiourea buffer. Thiourea buffer is usually used as soluble buffer for hydrophobic proteins[17]. Dowling *et al*[18] and Alvarez-Chaver *et al*[9] reported proteomic data of membrane proteins isolated from colorectal cancer by using Triton X-114. Although some common differentially expressed proteins were identified in our study and in the study by Alvarez-Chaver *et al*[9], there were also different types of proteins reported by both studies which indicated the crucial roles of reagents in determining the type of protein extracted.

Separation of proteins was carried out using 2-D gel electrophoresis. A similar quantity of proteins from cancerous and normal tissues was used in the analysis to ensure that the change in protein intensity was a factor of tissue type and not tissue weight, this is because the density of cancerous tissues is generally higher than that of normal tissues. A total of 500 μg of protein was shown to be an ideal load for 2-D gel separation as it allows the visualisation of minute proteins with good spot quality, while abundant proteins were kept under the saturation level when stained with Coomassie Blue.

In this study, only those proteins that were consistently expressed in all the tissues were targeted, we believe that this approach will minimise the identification of false-positive proteins that result from tissue heterogeneity and sample handling. In addition, the normalisation of protein spots during gel image analysis also serves as a control for the determination of differential protein expression levels. We observed a consistent pattern of proteomes for normal and cancerous tissues, respectively (Figure 1). Nevertheless, the expression levels of the proteins varied between patients, where no single protein was solely up-regulated or down-regulated in either cancerous or normal tissues in all patients; i.e. an un-regulated protein in one patient can be expressed as a down-regulated protein in another patient. This observation may support the phe-

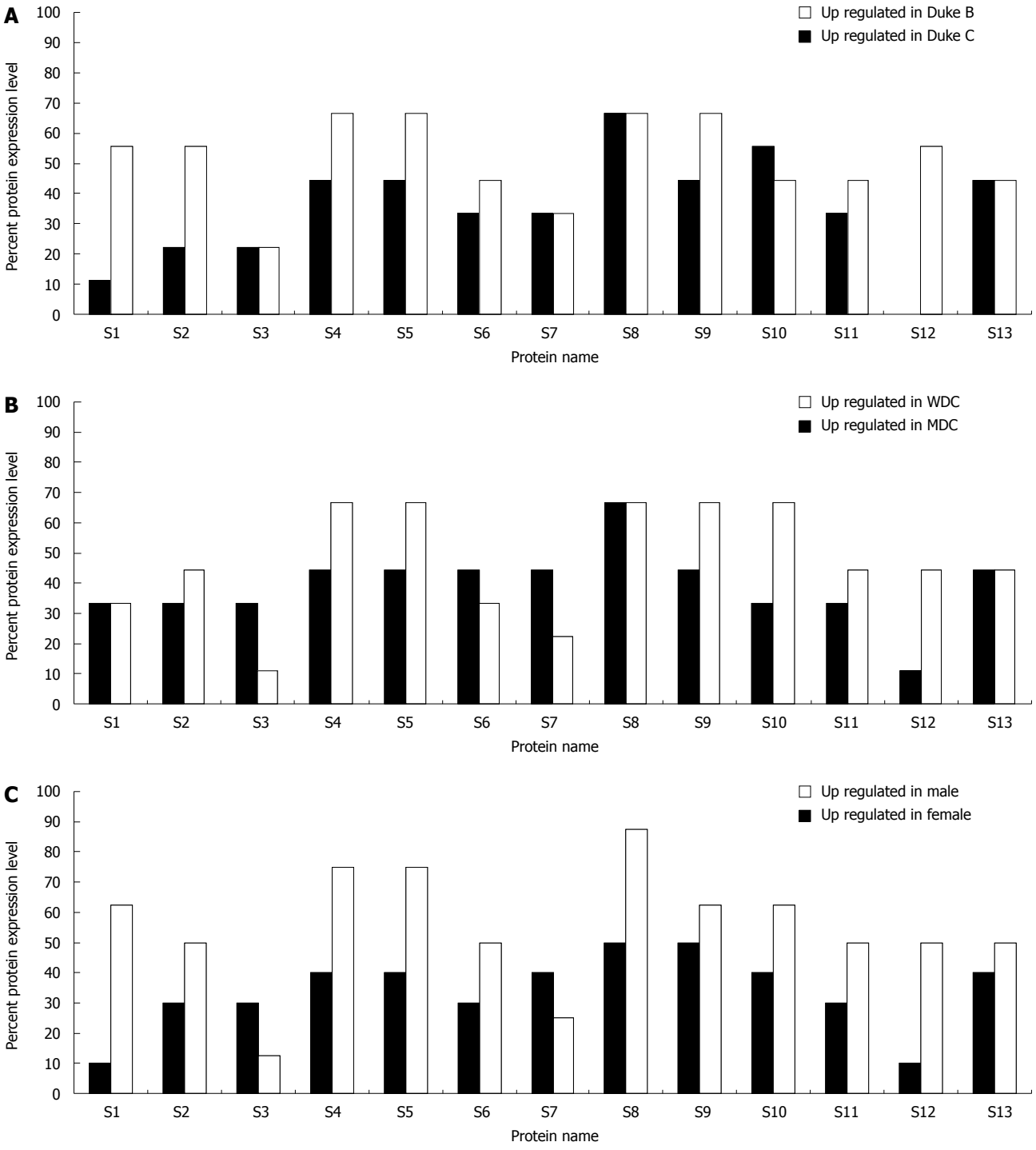


Figure 6 Distribution of proteins in cancerous tissues *vs* normal tissues in all patients in relation to cancer stage (A), grade (B) and gender (C). An up-regulated protein is a protein with a higher expression level in cancerous tissues than in normal tissues, and *vice versa* for down-regulated proteins.

nomenon that one drug does not fit all and therefore, customized drugs have become the current trend for the treatment of cancer. Nevertheless, protein expression level was found to be more consistent when the tissues were analysed according to cancer stage, grade and gender of the patients. The association of protein expression with stage and grade of CRC and gender of the patients showed the potential of developing stage, grade or gender specific treatment for CRC patients.

Duke's classification of tumor invasion has been proved to correlate with patient survival[19]. A greater number of proteins were found to be consistently up-regulated in Duke's C tissues compared to Duke's B tissues. In Duke's B cancer, the tumor has not yet metastasized and therefore may still undergo the process of differentiation, nevertheless, when it advances to Duke's C stage, where cancer has metastasized to the lymph nodes, the tumor may also reach a certain level of maturity and reveal

a greater consistency in the protein expression profile. In contrast, Kwong *et al*[20] reported that changes in the number of highly expressed proteins do not correlate with the progression of colorectal cancer from Duke's stage B to D.

Well-differentiated adenocarcinoma is less aggressive than moderately-differentiated adenocarcinoma, where the former has a greater resemblance to normal cells. We found that the pattern of protein expression in well-differentiated adenocarcinoma was closer to normal tissues, where only 1 protein was up-regulated in > 50% of well-differentiated tissues compared to 5 proteins in moderately-differentiated tissues.

With regard to the gender of patients, female patients showed a greater consistency in protein expression compared with male patients. In general, all 13 proteins identified had a much greater level of expression in female patients than in male patients except for heat shock 70 kDa protein and calreticulin. Calreticulin was identified as a down-regulated protein in this study. It has been reported that men have higher probabilities of developing polyps and tumors than women, although women are more likely to develop right-sided polyps and right-sided tumors than men[21], which may explain the different protein expression profiles in the two genders.

All 13 proteins identified in this study were among the highly expressed proteins in either cancerous or normal colorectal tissues. Of these proteins, SLP-2 has been shown to be a promising biomarker for CRC, particularly for female patients, where it was up-regulated in 87.5% of female patients, whereas in male patients, it was found to be up-regulated in 50% of patients. Moreover, in Duke's B, Duke's C, WDC and MDC, its up-regulation levels were 66.7%. Up-regulation of SLP-2 was statistically significant ($P < 0.05$) in both male and female patients and in the stages and grades of CRC tested. Its detection in CRC has not been reported, although it was reported to be up-regulated in other types of cancer. Over expression of SLP-2 was identified in human esophageal squamous cell carcinoma, lung cancer, laryngeal cancer, and endometrial adenocarcinoma which indicated that SLP-2 over expression is very common in cancer development. SLP-2 was associated with different stages of tumor progression from normal tissue to premalignant and malignant lesions of the esophagus[22]. High expression of SLP-2 was also attributed to advanced stages of breast cancer[23]. SLP-2 was reported to be involved in regulating cell growth and cell adhesion in human oesophageal squamous cell carcinoma[22]. SLP-2 plays an important role in sustaining T cell activation through the antigen receptor that is required for T cell differentiation during immunomodulation. It may also be involved in regulating ion channel conductance and/or the organisation of sphingolipid and cholesterol-rich lipid rafts[24].

The GRAVY score analysis of the thirteen proteins identified in this study showed that only prohibitin had a positive GRAVY score (0.024) indicating its hydrophobic nature. Nevertheless, Blonder *et al*[25] suggested that the GRAVY calculation does not reliably predict the hydrophobic nature of a protein. Therefore, the presence of the transmembrane domain of a protein is collectively used to predict the nature of a protein. Tmpred analysis of human SLP-2 (356 amino acids) has shown that it contains a single transmembrane domain, which putatively consists of 19 hydrophobic amino acids (amino acid 5-24). SLP-2 is a novel and unusual member of the stomatin gene superfamily. It is a peripheral membrane protein[24].

Besides SLP-2, other up-regulated proteins with significant expression were annexin A3, annexin A4 and tubulin β-2 chain. Annexin A3 and annexin A4 are members of the annexin family, a family of calcium-regulated phospholipid-binding proteins. Annexin A3 induces the migration and tube formation of vascular endothelial cells by inducing hypoxia-inducible factor-1 (HIF-1), which in turn causes the secretion of vascular endothelial growth factor (VEGF), an important factor in angiogenesis[26]. Annexin A4 forms complexes with protein kinase C, which has roles in cancer progression and was shown to be up-regulated in colorectal cancer[27]. Annexins have been reported to be involved in disease processes such as neoplasia. Furthermore, changes in the expression of annexins were linked with tumorigenesis[28]. Over expression of annexins in primary CRC increase significantly with advancing tumor stage, which suggests that annexins play a role in the progression and development of CRC[29]. Over expression of annexin A4 with advancing tumor stage was correlated with its role in promoting tumor cell migration. The distinct localization of annexin A4 in tumor cells was implicated to the loss of cell-to-cell adhesion and therefore increased tumor cell spread[30]. Our data showed that up-regulation of annexin A3 increased when the cancer stage advanced from Duke's B to Duke's C, while the opposite was observed for annexin A4. There have been no previous reports of annexin A3 participating in tumorigenesis or its association with different stages of CRC. Annexin A3 was reported to be up-regulated in colorectal tumor tissues and its cellular location was predominantly membrane-associated as revealed by immunohistochemistry assay[31]. Immunohistochemistry staining of annexin A3 in prostate cancer has shown its possible relationship with cancer grade[32]. In this study, we also observed that up-regulation of annexin A3 and annexin A4 in CRC increased when the cancer progressed from well-differentiated adenocarcinoma to moderately-differentiated adenocarcinoma.

The tubulin β-2 chain is one of the components of the cytoskeleton which plays a complex role in cells[33]. Significant differences in β-tubulin expression in polyps and invasive colon cancers indicates its possible roles in invasive cancer development[34]. A significant relationship between the expression of tubulins and stages of rectal cancer was suggested to be useful in identifying Dukes' B and Duke's C rectal cancer[34]. In this study, we found that the expression of tubulin β-2 chain increased significantly when the cancer advanced from Duke's B to Duke's C.

In conclusion, we have identified four hydrophobic proteins, namely SLP-2, tubulin β-2 chain, annexin A3

and annexin A4 which were abundantly expressed in cancerous tissues compared with normal tissues of the colon. Although a limited number of tissues were tested, the expression of these proteins in colorectal cancer was found to be significant suggesting that the possible use of these protein biomarkers in drug targeted therapy and in the diagnosis of colorectal cancer are worth further investigation.

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COMMENTS

Background

Colorectal cancer (CRC) is the third most common cancer worldwide. In Malaysia, the incidence of CRC is increasing. An evaluation of the protein profile of CRC tissues may lead to an understanding of the changes in protein expression when cancer progresses. Proteomics is an emerging tool to study proteins and therefore can be used for the identification of potential biomarkers in the detection and treatment of CRC.

Research frontiers

Membrane proteins comprise approximately 30% of the total human proteome. They are important component of cells and perform vital cellular functions. Due to their hydrophobic nature, many membrane proteins are difficult to extract and remain under-represented in protein profiles.

Innovations and breakthroughs

The authors have successfully extracted hydrophobic proteins from CRC patients. This group of proteins are differentially expressed and significantly correlated with stage and grade of cancer and gender of the patients. In their study, stomatin-like protein 2 (SLP-2) was significantly up-regulated in cancerous tissues. SLP-2 has not been previously reported in CRC.

Applications

Hydrophobic proteins are mainly located on the cell membrane. Therefore, they have the potential to be cell surface markers that can be used in drug targeted therapies for CRC.

Terminology

A hydrophobic protein is a protein that contains a stretch of amino acids that have hydrophobic side chains. These amino acids are arranged so that the hydrophobic side chains are placed outside the protein in the three dimensional structure of the protein. This part of the protein will tend to embed itself in lipid structures such as cell membranes.

Peer review

This manuscript describes the identification of a number of protein biomarkers that are up regulated in colorectal cancer. Membrane proteins from both cancer and normal mucosa were isolated using 2-D gel electrophoresis and subjected to LC/MS/MS analysis.

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ORIGINAL ARTICLE

Relationship between clinicopathological features and mucin phenotypes of advanced gastric adenocarcinoma

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Abstract

AIM: To investigate a relationship between the clinicopathological features and mucin phenotypes in advanced gastric adenocarcinoma (AGA).

METHODS: Immunohistochemical staining was performed to determine the mucin phenotypes in 38 patients with differentiated adenocarcinomas (DACs), 9 with signet-ring cell carcinomas (SIGs), and 48 with other diffuse-type adenocarcinomas (non-SIGs) of AGA. The mucin phenotypes were classified into 4 types: gastric (G), gastrointestinal (GI), intestinal, and unclassified.

RESULTS: The G-related mucin phenotypes were highly expressed in all the histological subtypes of AGA. The expression of the GI phenotype in SIG patients was lower than that in DAC patients (P = 0.02), and this phenotype was observed in 56% of the non-SIG patients in the intramucosal layer. Among non-SIG cases, the expression of the GI phenotype was significantly higher in patients with extended adenocarcinomas and those with positive rates of lymph node metastasis. There was no difference between the expressions of the G and other GI phenotypes factors. Among DAC and non-SIG patients, there were no differences between the survival rates of the corresponding patient groups.

CONCLUSION: The GI phenotype might possess more invasive characteristics than the G phenotype in non-SIG. Neither of the phenotypes indicated a poor prognosis of DAC and non-SIG.

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Key words: Mucins; Phenotype; Diffuse type; Undifferentiated type; Gastric neoplasms; Adenocarcinoma; Prognosis

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INTRODUCTION

Recently, an immunohistochemical method for mucin staining has been developed, and the expression of the mucin phenotype in gastric adenocarcinoma has been reported[1-4]. Lauren[5] and Nakamura *et al*[6] histologically classified gastric adenocarcinomas into 2 main types: in-

testinal type of Lauren's histology or differentiated type, and diffuse type or poorly differentiated type. The diffuse type adenocarcinomas, as classified using Lauren's method[7] (non-solid type of poorly differentiated adenocarcinoma and the signet-ring cell carcinoma according to a Japanese classification[8]: PDC), which do not show clustering or glandular formation, can be further divided into 2 histological subtypes. In one of these subtypes, the adenocarcinomas are predominantly (> 50%) composed of isolated malignant cells containing intracellular mucin and the nucleus is located at the periphery of the cytoplasm (signet-ring cell carcinoma: SIG according to the Japanese classification). In the other subtype, the adenocarcinoma contains few (< 50%) signet-ring cancer cells (non-SIGs in PDC).

On the basis of the differences in immunohistochemical staining, the mucin phenotypes in gastric adenocarcinoma can generally be divided into 4 types: gastric (G), mixed or gastrointestinal (GI), intestinal (I), and unclassified (UC). Some studies on the intestinal or differentiated types of adenocarcinoma have described the relationship between the expression of mucin phenotypes and the occurrence, progression, and clinicopathological features of adenocarcinomas[9-14]. The expression of the mucin phenotype in diffuse or poorly differentiated adenocarcinomas has also been studied[15-22]. However, some of these studies have been restricted to SIG[15-22], and there have been few studies on advanced gastric adenocarcinoma (AGA)[23-27]. Therefore, there is little information on the effects of the mucin phenotypes on the clinicopathological and histological subtype-based features of AGA, particularly in the diffuse type as defined by Lauren's method[7]. To clarify the role of the mucin phenotype in the clinicopathological features and prognosis of AGA, further studies should take into account the histological subtypes of AGA.

To this end, we examined the expression of mucin phenotypes in the above-mentioned histological subtypes of AGA. The mucin phenotype-based analysis was performed according to the classification proposed by Watanabe *et al*[28,29]. We also determined the relationship between the expression of the mucin phenotypes and the clinicopathological features, including the prognosis of patients, who were grouped according to the different histological subtypes of gastric adenocarcinoma.

MATERIALS AND METHODS

We investigated 95 subjects with AGA who had undergone gastric resection at the Surgery I Department of Gunma University Hospital between 1994 and 2000. Among the 95 subjects, 38 had differentiated adenocarcinoma (DAC), 9 had SIG, and 48 had non-SIG of PDC. We defined AGA as an adenocarcinoma that invades deep into the muscularis propria. The definition of DAC includes papillary and tubular adenocarcinomas classified using the Japanese classification[8]. The definitions of SIG and PDC are described above. The definition of non-SIG is the adenocarcinoma of NSC excluding SIG.

We used a staining method which had been previously reported[25,26]. In brief, the samples obtained after gastric resection were fixed in 10% buffered neutral formalin, macroscopically examined, and photographed. Thereafter, the resected tumor, which included the tumor center, was cut into 3-4 mm wide slices. These slices were then embedded in paraffin and stained with hematoxylin-eosin (HE). The slices were then examined and color images were used for the histochemical mapping of the tissues and for measuring tumor size. For immunohistochemistry, we selected 1 or 2 HE-stained sections obtained from the tumor areas with the largest diameters and the deepest mucosal invasion. Paraffin blocks containing the selected HE-stained sections were cut into consecutive 3 μm sections for immunohistochemical staining.

The following protocol was employed for staining. Deparaffinized sections were treated with citrate buffer (pH 6.0), heated in a microwave oven for 20 min, and allowed to be cooled to room temperature. Endogenous peroxidase activity was blocked by incubating the sections for 20 min with 0.3% hydrogen peroxidase in absolute methanol, and the sections were then washed in tap water. Non-specific binding was blocked by using normal serum (Nichirei, Japan). The sections were incubated with a primary antibody overnight at 4℃ and then incubated with a biotinylated secondary antibody for 30 min at room temperature (Nichirei, Japan). Immunohistochemical staining was performed using a streptavidin-biotin-peroxidase kit (Nichirei, Japan) according to the manufacturer's instructions, with slight modifications. We used MUC5AC (diluted 1:100; antibody CLH2, Novocastra, UK) and human gastric mucin (HGM, diluted 1:50; antibody 45M1, Novocastra) antibodies as markers for the gastric foveolar phenotype; MUC6 (diluted 1:100; antibody CLH5, Novocastra) and M-GGMC-1 (diluted 1:50; antibody HIK1083, Kantou Chemicals, Japan) antibodies as markers for the pyloric gland phenotype; MUC2 (diluted 1:500; antibody Ccp58, Novocastra) as a marker for intestinal goblet cell mucin; and CD10 (diluted 1:200; antibody 56C6, Novocastra) as a marker for small intestinal enterocytes.

The markers HGM, M-GGMC-1, and CD10 exhibited both cytoplasmic and luminal membranous reactivity, whereas MUC5AC, MUC6, and MUC2 exhibited only cytoplasmic reactivity. The reactivity was estimated on the basis of the percentage of stained cells among the total number of tumor cells in each stained section. On the basis of the frequency of positive staining for the relevant marker, the adenocarcinoma phenotypes were classified into 4 groups: G, GI, I, and UC. The following criteria were used for the classification of the mucin phenotypes: (1) if more than 5% of the cells were positive for HGM, MUC5AC, MUC6, or M-GGMC-1, the phenotype was classified as G; (2) if more than 5% of the cells were positive for MUC2, the phenotype was classified as I; and (3) if even a single cell was positive for CD10, the phenotype was classified as I. If none of the above criteria were met, the phenotype was classified as UC. The mucin phenotype was determined by examining the intramucosal layer.

Table 1 Clinical profiles and tumor characteristics according to the histological subtypes

Histological subtype	Gender		Age at operation (yr)	Tumor site			Tumor size (cm)	
	Male	Female		U	M	L	< 5	> 5
DAC (n = 38)	28	10	58	9	12	17	17	21
SIG (n = 9)	3[a]	6[a]	62	2	1	6	3	6
Non-SIG (n = 48)	24	24	65	7	27	14	14	34

DAC: Differentiated-type adenocarcinoma; SIG: Signet-ring cell carcinoma; Non-SIG: Other diffuse-type adenocarcinomas; U: Upper segment of the stomach; M: Middle segment of the stomach; L: Lower segment of the stomach. [a]P = 0.05.

Table 2 Expression rates of the various mucin phenotypes at the intramucosal layer among patients classified according to the histological subtype

Antigen/histological subtype	HGM (%)	MUC5AC (%)	MUC6 (%)	M-GGMC-1 (%)	MUC2 (%)	CD10 (%)
DAC (n = 38)	97	95	84	90[a,b]	76[c]	0
SIG (n = 9)	100	100[d]	56	44[a,d]	33[c]	0
Non-SIG (n = 48)	96	98[e]	65	52[b,e]	56	0

HGM: Human gastric mucin. [a]P < 0.01, [b]P < 0.01, [c]P = 0.02, [d]P = 0.02, [e]P < 0.01.

In addition to determining the mucin phenotype, we reviewed the patients' profiles (age at operation and gender), the tumor site (upper segment of the stomach, U; middle segment of the stomach, M; and lower segment of the stomach, L), the tumor size, the histological findings (lymphatic and vessel invasion, metastasis to the lymph nodes and other sites), and patient survival. The relationships among these factors, particularly the comparison between the G and GI phenotypes, were determined.

The survival rates of the patients with G and GI phenotypes grouped according to the histological subtypes were examined by Kaplan-Meier analysis.

Statistical analysis was performed using the Pearson χ^2 test [Fisher's exact test (extended)] and the Student t-test. Two-tailed P-values < 0.05 were considered significant.

This work has been carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association and all the participants gave their written informed consent.

RESULTS

Clinical profiles and tumor characteristics of the patients with DAC, SIG, and non-SIG adenocarcinomas

The clinical profiles and tumor characteristics of the patients with DAC, SIG, and non-SIG are summarized in Table 1. Among women, the number with SIG was significantly higher than those with DAC (P = 0.05). There were no intergroup differences in the age at operation or size of tumor. For the site of tumor, the number in the lower segment in DAC was higher than that in SIG or DAC.

Typical photographs of a patient with SIG expressing the G phenotype and of a patient with non-SIG expressing the GI phenotype

Typical photographs of a patient with SIG expressing the G phenotype are shown in Figure 1. Immunohistochemical staining revealed a large number of HGM- or M-GGMC-1-positive cells in the mucosal portion of the tumor. No MUC2-positive cells were detected in the tumor area. The cancer cells, most of which were signet-ring cells, were scattered without clustering or glandular formation.

Figure 2 shows typical photographs of a patient with non-SIG expressing the GI phenotype. Immunohistochemical staining revealed a number of M-GGMC-1-positive cells in the mucosal portion of the tumor. We also observed sporadically distributed MUC2-positive cells. No HGM-positive cells were detected in the tumor area of the examined tissues. We observed scattered non-SIG cells similar to the SIG shown in Figure 1. No cluster and glandular formation of cancer cells was detected in any region of tumor.

Expression rates of the various mucin phenotypes at the mucosal layer among patients classified according to the histological subtypes

The expression rates of various mucin phenotypes at the mucosal layer among the patients classified according to the histological subtype are shown in Table 2. The expression rates of HGM and MUC5AC were higher than those of MUC6 and M-GGMC-1 in all the histological subtypes, particularly in the cases of SIG (P = 0.02) and non-SIG (P < 0.01). The expression rate of M-GGMC-1 in the cases of DAC was higher than that in the cases of SIG (P < 0.01) and non-SIG (P < 0.01).

The MUC2-expression rates in the cases of DAC, SIG, and non-SIG were 76%, 33%, and 56%, respectively. The MUC2-expression rate was lowest in the cases of SIG, and the expression rate in the cases of DAC was higher than that in the cases of SIG (P = 0.02) and non-SIG (P = 0.06). No CD10 expression was detected in any of the cases.

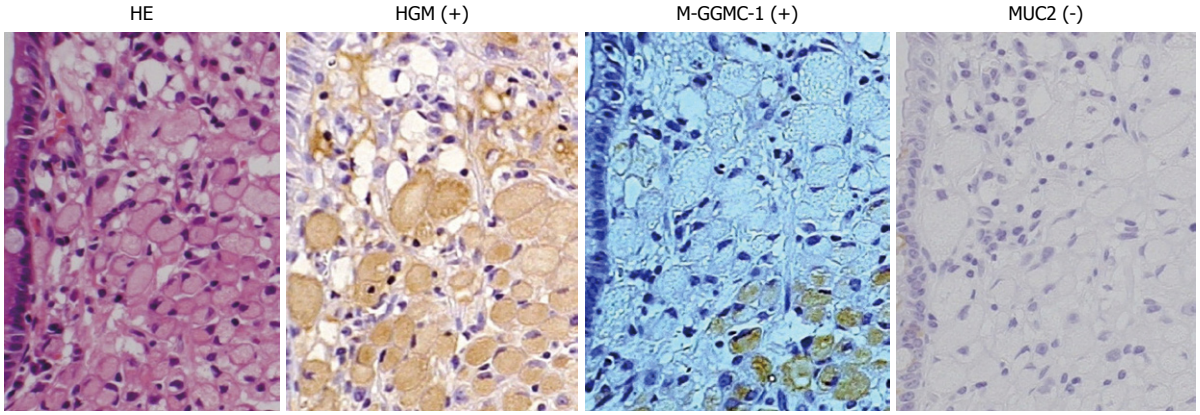


Figure 1 Typical photographs of a patient with signet-ring cell carcinoma (SIG) expressing the gastric (G) phenotype.

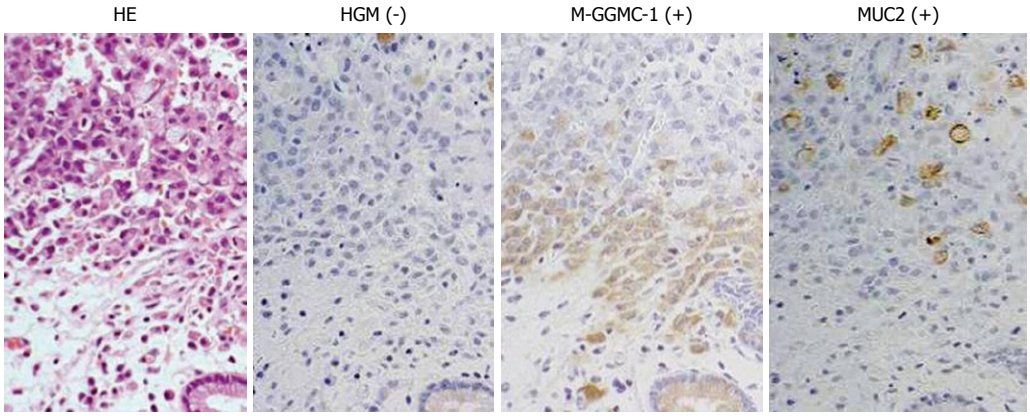


Figure 2 Typical photographs of a patient with non-SIG expressing the gastrointestinal (GI) phenotype.

Table 3 Expression rates of the various mucin phenotypes at the intramucosal layer of gastric adenocarcinomas according to the histological subtypes *n* (%)

Mucin phenotype/ histological subtype	G	GI	I	UC
DAC (n = 38)	9/38 (24)	29/38 (76)[a,b]	0/38 (0)	0/38 (0)
SIG (n = 9)	6/9 (67)	3/9 (33)[a]	0/9 (0)	0/9 (0)
Non-SIG (n = 48)	19/48 (40)	27/48 (56)[b]	0/48 (0)	2/48 (4)

G: Gastric phenotype; GI: Gastrointestinal phenotype; I: Intestinal phenotype; UC: Unclassified phenotype. [a]P = 0.02; [b]P = 0.06.

Expressions of G, GI, I, and UC phenotypes at the mucosal layer among patients classified according to the histological subtypes

The expressions of the G, GI, I, and UC phenotypes at the mucosal layer among the patients classified according to the different histological subtypes are shown in Table 3. The expressions of the G, GI, I, and UC phenotypes in patients with DAC were 24%, 76%, 0%, and 0%, respectively; the expressions in patients with SIG were 67%, 33%, 0%, and 0%, respectively; and the expressions in patients with non-SIG were 40%, 56%, 0%, and 4%, respectively. For all the histological subtypes, the expressions of the I and UC phenotypes were extremely low and those of the G and GI phenotypes were extremely high. The expression of the GI phenotype in the cases of DAC was higher than that in the cases of SIG (P = 0.02) and non-SIG (P = 0.06).

Comparison between the expressions of the G and GI phenotypes with respect to clinical and histological findings in patients classified according to the histological subtypes

Table 4 presents a comparison between the expressions of the G and GI phenotypes with respect to the clinical profiles, tumor site, tumor size, metastasis rate, and histological findings among the patients classified according to the histological subtypes. In SIG, all 6 female patients expressed the G phenotype and all 3 male patients expressed the GI phenotype (P = 0.01). Among the subjects with non-SIG, the number of GI phenotype cases with a tumor diameter greater than 5 cm was significantly higher than the corresponding number of G phenotype cases (P = 0.01). The positive rate of lymph node metastasis in the GI phenotype cases was also significantly higher than the corresponding value in the G phenotype cases (P = 0.01). There were no significant differences in other factors between the patients expressing the G and GI phenotype.

Table 4 Comparisons between the expression of G and GI phenotypes in clinical profiles, tumor site and tumor size, metastasis rate, and histological findings, according to the histological subtype

Histological subtype	DAC		SIG		Non-SIG	
Mucin phenotype	G (n = 9)	GI (n = 29)	G (n = 6)	GI (n = 3)	G (n = 19)	GI (n = 27)
Male:female	6:3	12:17	0:6[a]	3:0[a]	9:10	10:17
Mean age (yr)	57.2	60.3	63.2	55.3	68.2	60.9
Tumor site						
U	2	7	2	0	2	5
M	2	10	0	1	9	18
L	5	12	4	2	8	4
Tumor size (cm)						
< 5	3	17	3	0	9[b]	3[b]
> 5	6	12	3	3	10[b]	24[b]
Metastasis rate (expression rate, %)						
Lymph node	67	45	83	100	42[c]	81[c]
Other site	22	3	50	33	11	19
Histological findings (expression rate, %)						
Vessel invasion	67	69	67	100	84	93
Lymph invasion	100	86	83	100	95	100

[a]P = 0.01, [b]P = 0.01, [c]P = 0.01.

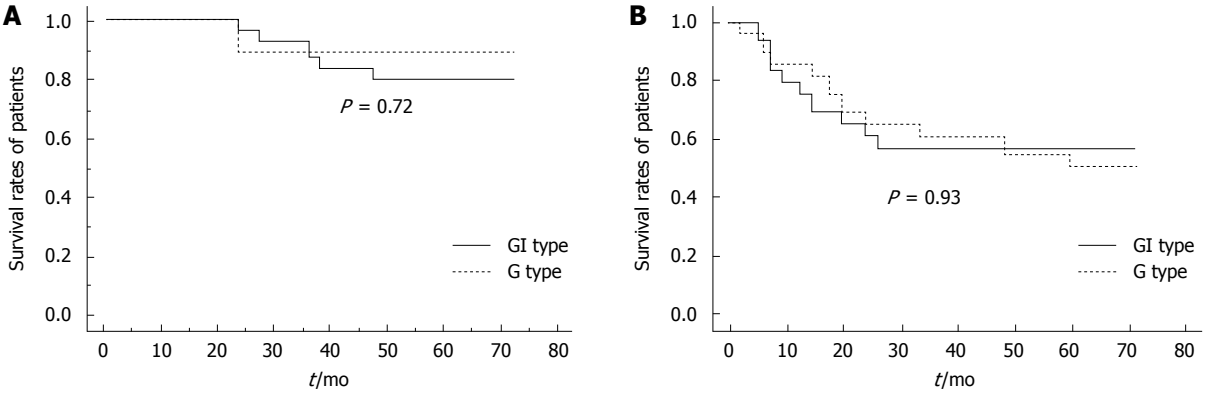


Figure 3 Survival rates of patients with the G and GI phenotypes in differentiated adenocarcinoma (DAC) (A) and non-SIG (B). The Kaplan-Meier method was used for the analysis.

Survival rates of the patients expressing the G and GI phenotype classified according to the histological subtypes

The survival rates of the G and GI phenotype cases classified according to the histological subtypes are shown in Figure 3. Among the patients with DAC, there was no significant difference between the survival rates of patients expressing the G phenotype and those expressing the GI phenotype (Figure 3A). The survival rates among the non-SIG patients expressing the G phenotype and those expressing the GI phenotype (Figure 3B) were also not significantly different (P = 0.93).

DISCUSSION

In the present study, HGM and MUC5AC were highly expressed in all histological subtypes. Among the SIG and non-SIG cases examined, the expression rates of MUC6 and M-GGMC-1 were lower than those of HGM and MUC5AC. The expression rates of MUC6 and M-GGMC-1 in the DAC were higher than those in the SIG and non-SIG cases. Pinto-de-Sousa *et al*[18] showed that the mucin phenotype is associated with the tumor site. In the SIG and non-SIG cases in the present study, the upper or middle segments of the stomach were the most common tumor sites. The differences between the rates of expression of the G-related phenotypes were believed to have been influenced by the tumor location. In addition, most cases of AGA showed the presence of gastric foveola phenotype. Further, we observed that the proportion of women in the SIG group was high and that none of the women with SIG expressed the GI phenotype. With regard to the clinical features of the mucin phenotype, the SIGs in women had specific clinical features.

On the basis of the examination of various mucin phenotype-expressing antigens, we classified almost all the AGAs (over 95%) as having either a G or GI phenotype. Furthermore, we failed to detect the expression of the pure I phenotype in any of the cases. The expression rate of the UC phenotype was also extremely low. Pinto-de-Sousa *et al* studied the mucin phenotypes of 23 diffuse-type adenocarcinomas classified according to Lauren's method[7] and showed that the MUC5AC-ex-

pression rate in these adenocarcinomas was significantly higher than that in the unclassified and expansive adenocarcinomas[18]. Further, Reis *et al*[4] studied the expression of MUC5AC in early gastric adenocarcinomas and suggested that all gastric adenocarcinomas retain at least some G phenotype cells in the initial stages of neoplasm development. Therefore, we postulate that almost all the cases of advanced DAC, SIG, and non-SIG exhibit or retain the features of G-related phenotypes in the intramucosal layer.

In the present study, the I phenotype (GI phenotype) was expressed in 56% of the non-SIG cases. Barresi *et al*[21] reported that 8 out of every 10 cases of diffuse-type adenocarcinomas were MUC2-positive. Tajima *et al*[23] studied the expression rates of the GI and I phenotypes in the cases of undifferentiated type AGA and reported that the I phenotype was expressed in half of the cases. Yamachika *et al*[15] and Bamba *et al*[16] reported that the progression of SIG was associated with a phenotypic shift from the G-type to the I-type expression. Other studies have described the invasive features of the I phenotype in gastric adenocarcinoma[19,22,26]. The results of our study and the abovementioned studies suggest that more than 50% of the cases with non-SIG AGA exhibit the features of the I phenotype and that this phenotypic feature is acquired or transformed during the initial progression stage of these adenocarcinomas. In addition, our results indicate that the GI phenotype is associated with invasive features.

The expression rate of the I phenotype (GI phenotype) among the SIG cases was 33% and the number of SIG cases was low (9 cases). We assume that SIG cells with the G phenotype cannot progress to the deep layer. It is also suggested that the morphological features of the SIG cells change and are subsequently classified as non-SIG during tumor progression.

In the present study, we also examined the relationships between the expression of mucin phenotypes and the clinicopathological features and prognosis of patients with DAC, SIG, and non-SIG. Among the non-SIG cases, the expression rate of the GI phenotype in both the patients with extended tumors and those with lymph node metastasis was significantly high. The survival rates of the DAC and non-SIG patients who expressed the G or GI phenotypes were not significantly different. These results indicate that the acquisition of the I phenotype in patients with non-SIG AGA is related to tumor extension and lymph node metastasis and that the existence or acquisition of this phenotype does not affect the survival rate of these patients. Our results indicate that the GI phenotype is associated with invasive features as described above. In SIG, the relationship between the survival rates of G and GI phenotype patients is unknown due to the small number of patients with these phenotypes.

The high rate of lymph node metastasis and the invasive tendency of the non-SIG cells with the GI phenotype may influence the 5-year survival rate, and the patients with the GI phenotype may thus show poor prognosis. However, in the present study, there was no difference between the survival rates of G and GI phenotype patients showing these features. With regard to the features of the mucin phenotype in differentiated-type gastric adenocarcinoma, the G phenotype has been reported to have malignant features[12,13]. In contrast to our study, Tajima *et al*[23] studied the expression of the mucin phenotype in patients with AGA and reported that patients with the G phenotype adenocarcinomas had a poorer outcome than those with the I phenotype adenocarcinomas. Mizoshita *et al*[24] reported that AGA patients expressing the GI phenotype had a relatively good prognosis. Although the reason for the difference is unclear, it could be attributed to the difference in the histological subtypes of the examined adenocarcinomas; this is because almost all the studies reporting the malignant potential of the G phenotype have considered differentiated adenocarcinomas[12,13]. Further, we mainly studied the diffuse type of AGA, which was classified into the SIG and non-SIG types, namely the restricted diffuse type of Lauren's classification. In addition, we assume that the prognosis of the patients with advanced non-SIG will also depend on postoperative treatments such as postoperative chemotherapy.

Therefore, we assume that the expression of the G or GI phenotypes in cases of the advanced non-SIG type of pure diffuse-type gastric adenocarcinoma does not indicate a poor prognosis during the 5-year postoperative period.

In conclusion, the GI phenotype showed a high expression rate (56%) in patients with advanced non-SIG, thereby indicating the acquisition of I phenotypic features during the progression of adenocarcinomas. In patients with advanced non-SIG, although the GI phenotype may be associated with greater invasiveness than the G phenotype, the survival rates of patients expressing either phenotype are similar, suggesting that neither the G nor the I phenotype indicates a poor prognosis in this type of adenocarcinoma. However, the presence of the I phenotype in patients with advanced SIG is unknown.

COMMENTS

Background

The relationship between the clinicopathological features and mucin phenotypes in advanced gastric adenocarcinoma (AGA) classified according to the histological subtype [differentiated adenocarcinoma (DAC), signet-ring cell carcinoma (SIG), and diffuse-type adenocarcinoma (non-SIG)] is unclear.

Research frontiers

Recently, the expression of the mucin phenotype in gastric adenocarcinoma was reported. Lauren and Nakamura histologically classified gastric adenocarcinomas into 2 main types: intestinal type of Lauren's histology or differentiated type, and diffuse type or poorly differentiated type. There is little information on the effects of the mucin phenotypes on the clinicopathological and histological subtype-based features of AGA, particularly in the diffuse type as defined by Lauren's method.

Innovations and breakthroughs

This study is the first report that investigated the mucin phenotype in advanced differentiated, SIG and non-SIG gastric adenocarcinomas. The GI phenotype might possess more invasive characteristics than the G phenotype in non-SIG patients. However, neither of the phenotypes indicates a poor prognosis of DAC and non-SIG.

Applications

Mucin phenotypes of gastric adenocarcinoma are related to the biological features in these cancers. The study of mucin phenotype is useful to clarify the biological features in gastric adenocarcinoma.

Terminology

Mucins of stomach are heavily glycosylated glycoproteins that are the major components of the mucous viscous gel covering gastric surface mucous cells, pyloric gland cells, intestinal goblet cells of the mature gastrointestinal tract, and the brush border of intestinal epithelial cells, *etc*.

Peer review

The data presented are interesting and helpful for further understanding the possible clinical value of mucin phenotypes of gastric carcinomas. The conclusions extracted are reasonable, although most of them are disproving.

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ORIGINAL ARTICLE

Protective effect of recombinant human IL-1Ra on CCl_4-induced acute liver injury in mice

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Abstract

AIM: To evaluate the effects of positive regulation of recombinant human interleukin 1 receptor antagonist (rhIL-1Ra) on hepatic tissue recovery in acute liver injury in mice induced by carbon tetrachloride (CCl_4).

METHODS: Acute liver damage was induced by injecting 8-wk-old mice with CCl_4 1 mL/kg (1:3 dilution in corn oil) intraperitoneally (ip). Survival after liver failure was assessed by injecting 8-wk-old mice with a lethal dose of CCl_4 2.6 mL/kg (1:1 dilution in corn oil) ip. Mice were subcutaneously injected with 1 mg/kg recombinant human IL-1Ra twice a day after CCl_4 treatment for 5 d. Serum alanine amino transferase (ALT) and aspartate aminotransferase (AST) levels were determined with a commercial assay kit. Serum IL-1β, IL-1Ra levels were measured by enzyme-linked immunosorbent assay kit. Quantitative real-time polymerase chain reaction was used to determine liver IL-1β, IL-1Ra and IL-6 expression during CCl_4-induced acute liver injury. Liver sections were stained with hematoxylin-eosin. A histology-injury grading system was used to evaluate the degree of necrosis after acute liver injury. Proliferating cell nuclear antigen (PCNA) staining was used to evaluate the role of rhIL-1Ra in promoting hepatocyte proliferation.

RESULTS: Quantitative analysis showed a higher level of IL-6 mRNA expression and reduced serum AST and ALT levels in the livers of the rhIL-1Ra-treated group at the early phase of CCl_4-induced acute liver injury. Histological examination indicated a decrease in centrilobular necrotic areas in mice treated with rhIL-1Ra, and a novel role of rhIL-1Ra in promoting hepatocyte proliferation was also supported by an increase of PCNA staining. All these results, accompanied by a strong survival benefit in rhIL-1Ra-treated *vs* PBS-treated groups, demonstrated that rhIL-1Ra administration ameliorated the histological damage and accelerated the regeneration and recovery process of the liver.

CONCLUSION: rhIL-1Ra could be further developed as a novel therapeutic agent for the treatment of acute liver injury because of its ability to reduce hepatocellular damage and facilitate liver regeneration.

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Key words: Recombinant human interleukin 1 receptor antagonist; Carbon tetrachloride; Liver injury; Hepatocyte proliferation

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INTRODUCTION

The interleukin (IL)-1 family includes the structurally related proteins IL-1α, IL-1β, and interleukin 1 receptor antagonist (IL-1Ra) that bind to the same cell surface receptor. However, IL-1Ra functions as a competitive inhibitor of IL-1α and IL-1β[1]. IL-1α and IL-1β play a key role in inflammation[2]; in response to stimuli such as inflammatory agents, infections, or microbial endotoxins, a dramatic increase of IL-1 mediated by macrophages and various other cell types is seen[3,4]. IL-1Ra is a naturally occurring anti-inflammatory protein; it competitively blocks the binding of IL-1α and IL-1β to type I IL-1 receptor, but exerts no agonist activity[5,6]. IL-1Ra has been shown to inhibit the effects of IL-1 both *in vitro* and *in vivo*, and as an acute-phase protein, it can reduce the severity of several animal models of inflammatory disease[7]. Serum levels of IL-1Ra can rise dramatically during different inflammatory and noninflammatory conditions[8]. Human IL-1Ra is synthesized as a 177 amino acid precursor that contains a 25 amino acid signal sequence plus a 152 amino acid mature region[9,10].

Acute administration of carbon tetrachloride (CCl_4) is used to establish an experimental model of severe hepatocellular damage involving generation of oxidative stress and recruitment of inflammatory cells[11-13], which is reported to induce liver architectural and functional damage[14-16]. Liver regeneration involves a complex regulated response to CCl_4-induced acute liver injury[17,18].

In this study, we aimed to examine the effects of IL-1Ra as an acute phase protein in reducing hepatic injury and accelerating hepatocyte proliferation following CCl_4 administration. Although the anti-inflammatory effect of IL-1Ra has been described, the contribution of this cytokine to protect from the liver injury remains unclear. The present findings indicate that IL-1Ra is a critical factor that shows a potent antihepatotoxic activity in recovery of hepatocellular necrosis and in acceleration of liver regeneration during injury. IL-1Ra could provide a novel therapeutic approach by stimulating liver regeneration.

MATERIALS AND METHODS

Animals

Procedures were performed in male C57BL/6 mice (purchased from SLC Shanghai, China) 8 wk after birth, maintained in a conventional clean facility and in accordance with the National Animal Care and Use Committee.

Cytokine and reagents

Recombinant human IL-1Ra (rhIL-1Ra) was obtained from Dr Wei Han's Laboratory at the School of Pharmacy, Shanghai Jiao Tong University. Endotoxin level of the rhIL-1Ra was under 0.1 EU/μg. CCl_4 was purchased from Sigma, USA.

Acute liver injury and lethal dose performance

Acute liver injury was induced by injecting 8-wk-old mice with CCl_4 1 mL/kg (1:3 dilution in corn oil) intraperitoneally (ip). A lethal dose was administered by injecting 8-wk-old mice with CCl_4 2.6 mL/kg (1:1 dilution in corn oil) ip.

rhIL-1Ra and PBS injection

Mice were subcutaneously injected with 1 mg/kg rhIL-1Ra (diluted to 0.5 mg/mL with PBS) twice a day after CCl_4 administration for 5 d because human and murine IL-1Ra show an overall homology of 77% with no apparent species specificity[19]. The first rhIL-1Ra injection was performed at 1 h after CCl_4 treatment. The control group of mice was subcutaneously injected with the same volume of PBS.

Serum aspartate aminotransferase and alanine amino transferase

Serum aspartate aminotransferase (AST) and alanine amino transferase (ALT) levels were determined with a commercial assay kit (Nanjing Jiancheng Biological Technology, Inc., China). Enzyme activities were expressed as an international unit per liter (IU/L).

Enzyme-linked immunosorbent assay

Serum IL-1β and IL-1Ra level were measured by enzyme-linked immunosorbent assay (ELISA) kit (R&D system, Minneapolis, MN, USA) according to the manufacturer's instructions.

Histology-injury grading

Formalin-fixed, paraffin-embedded liver sections were stained with hematoxylin-eosin for the histological investigations. To evaluate the degree of necrosis after acute liver injury we created an injury grading score (Grade I - IV) based on severity of necrotic lesions in the liver parenchyma (Table 1).

Proliferating cell nuclear antigen staining

For proliferating cell nuclear antigen (PCNA) immunohistochemical staining, de-paraffinized sections of liver blocks were used. Liver tissues were fixed for 24 h in neutral buffered formalin, processed routinely and embedded in wax. Immunohistochemical staining was performed as previously described[16]. The sectioned liver tissues were stained using a mouse monoclonal antibody against PCNA and the SABC Staining Kit (Wuhan Boster Biological Technology, Wuhan, China) according to manufac-

Table 1 Injury grade

No. of mice	Day + 2[1]	Day + 3[1]	Day + 5[1]	Day + 7[1]
Group A (rhIL-1Ra)				
1	Ⅲ	Ⅱ	Ⅰ	0
2	Ⅲ-Ⅳ	Ⅰ	Ⅰ	0
3	Ⅲ	Ⅰ	0	0
4	Ⅲ	Ⅱ	Ⅰ	0
5	Ⅲ-Ⅳ	Ⅰ	0	Ⅰ
6	Ⅳ	Ⅰ	0	0
Group B (PBS)				
1	Ⅲ-Ⅳ	Ⅱ-Ⅲ	Ⅰ-Ⅱ	0
2	Ⅳ	Ⅲ	Ⅱ	Ⅰ
3	Ⅲ-Ⅳ	Ⅲ	Ⅱ	0
4	Ⅳ	Ⅱ-Ⅲ	Ⅱ	0
5	Ⅳ	Ⅲ	Ⅱ	Ⅰ
6	Ⅲ-Ⅳ	Ⅲ	Ⅰ	Ⅰ

[1]Day of sacrifice after CCl_4 1 mL/kg (1:3 dilution in corn oil) ip treatment. Injury grading with respect to severity of necrosis in liver parenchyma: Grade 0: Normal histology; Grade Ⅰ: Presence of degenerated hepatocytes with only rare foci of necrosis; Grade Ⅱ: Mild centrilobular necrosis around the central vein, occupying only a part of Rappaport's zone Ⅲ; Grade Ⅲ: Established necrosis limited to zone Ⅲ; Grade Ⅳ: Extensive, confluent centrilobular necrosis involving Rappaport's zone Ⅲ and Ⅱ. rhIL-1Ra: Recombinant human interleukin 1 receptor antagonist.

turer's protocol, then subjected to photomicroscopic observation (NIS-Elements Basic Research, Nikon Eclipse 50i, Kanagawa, Japan).

Quantitative real-time polymerase chain reaction

Total RNA was obtained from the liver of mice and was prepared using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). The quantification and qualification of RNA were determined by UV absorbance and electrophoresis in 1.2% agarose. RNA quality was satisfied when the 28s rRNA banding was twice the intensity of the 18s rRNA without significant smearing of the rRNA bands. Quantitative real-time polymerase chain reaction (RT-PCR) reactions were performed with the MJ chromo 4 RT-PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA). Specific primers were designed using Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA) and their sequences are listed as follows: IL-1β (sense) 5'TGAGCACCTTCTTTTCCTTC3', IL-1β (anti-sense) 5'GTTCATCTCGGAGCCTGTAG3'; IL-1Ra (sense) 5'AGACCTTGTGTCCTGTTTAGC3', IL-1Ra (anti-sense) 5'GGTCAATAGGCACCATGTCT3'; IL-6 (sense) 5'CCACTCCCAACAGACCTGTCTATAC3', IL-6 (anti-sense) 5'CACAACTCTTTTCTCATTTCCACGA3'; β-actin (sense) 5'AGCCTTCCTTCTTGGGTATG3', β-actin (anti-sense) 5'GTGTTGGCATAGAGGTCTTTAC3'. For the RT-PCR reaction, the following procedure was followed. Total RNA (5 μg) was used as a template for synthesizing the first-strand of cDNA with M-MuLV reverse transcriptase (MBI Fermentas, Vilnius, Lithuania) in a 20 mL reaction volume. PCR reactions were carried out by adding 100 × diluted cDNAs, 100 nmol/L of each primer, and SYB Premix Ex *Taq* (TaKaRa, Dalian, China) in 20 μL reactions. PCR conditions were optimized using Opticon monitor 3 software (Bio-Rad Laboratories) and involved the following steps: 95℃ for 5 min, 1 cycle; 95℃ for 5 s and 60℃ for 30 s, 40 cycles. Final data were analyzed with Opticon monitor 3 software (Bio-Rad Laboratories), presented as ratios to β-actin for each time point.

Statistical analysis

Results are expressed as mean ± SD. Statistically significant differences over time in the same treatment group, or among different treatment groups at a single time point, were determined by Student's *t* test. $P < 0.05$ was considered to be statistically significant. Results from survival experiments were analyzed using the log-rank test and expressed as Kaplan-Meier survival curves.

RESULTS

IL-1β, IL-1Ra and IL-6 expression during CCl_4-induced acute liver injury

Expression of IL-1β mRNA decreased in the first 12 h, and reached its lowest point at day 1.5 (Figure 1A). In contrast, expression of IL-1Ra mRNA was rapidly induced and reached a peak within 12 h following 1 mL/kg CCl_4 administration (Figure 1B). Serum level of IL-1β did not increase so rapidly (Figure 1C). We found that serum IL-1Ra enhanced markedly after CCl_4 administration (Figure 1D), induced by generation of oxidative stress and recruitment of inflammatory cells. We confirmed that an adequate ratio of serum IL-1Ra to IL-1 was crucial to the recovery of liver injury (Figure 1E), and we found that the ratio reached a peak at day 1.5 after CCl_4 administration. Furthermore, the expression of IL-6 mRNA was also stimulated by excessive treatment with rhIL-1Ra (Figure 1F).

rhIL-1Ra protects mice from acute hepatocellular damage

CCl_4-induced acute liver injury that results in a quantifiable liver damage recovers naturally within 7 d, as mice sacrificed 7 d after CCl_4 injection appear with normal liver histology. To confirm the role of rhIL-1Ra in protecting from hepatic damage, we investigated the effect of rhIL-1Ra on CCl_4-induced acute liver injury. Mice were subcutaneous injected with rhIL-1Ra and PBS after CCl_4 administration. Animals were sacrificed 1, 3, 5 and 7 d after CCl_4 administration for AST and ALT determination. The serum level of ALT or AST rapidly elevated to reach a peak at day 1 then decreased thereafter in PBS-treated control mice, while rhIL-1Ra treatment significantly inhibited the elevation of ALT and AST from day 1 to day 5 (Figure 2A and B). The reduction of serum AST and ALT indicated that rhIL-1Ra has a direct protective effect on hepatocytes. To evaluate the effect of rhIL-1Ra on hepatocellular necrosis and inflammation, histological changes in the liver after CCl_4 administration with or without rhIL-1Ra treatment were examined by histology-injury grading (Table 1). Liver sections from PBS-treated animals showed hepatocellular necrosis and inflammation at day 3 after CCl_4 administration; in contrast, liver sections from the rhIL-1Ra-treated group demonstrated only mild hepatocellular necrosis and in-

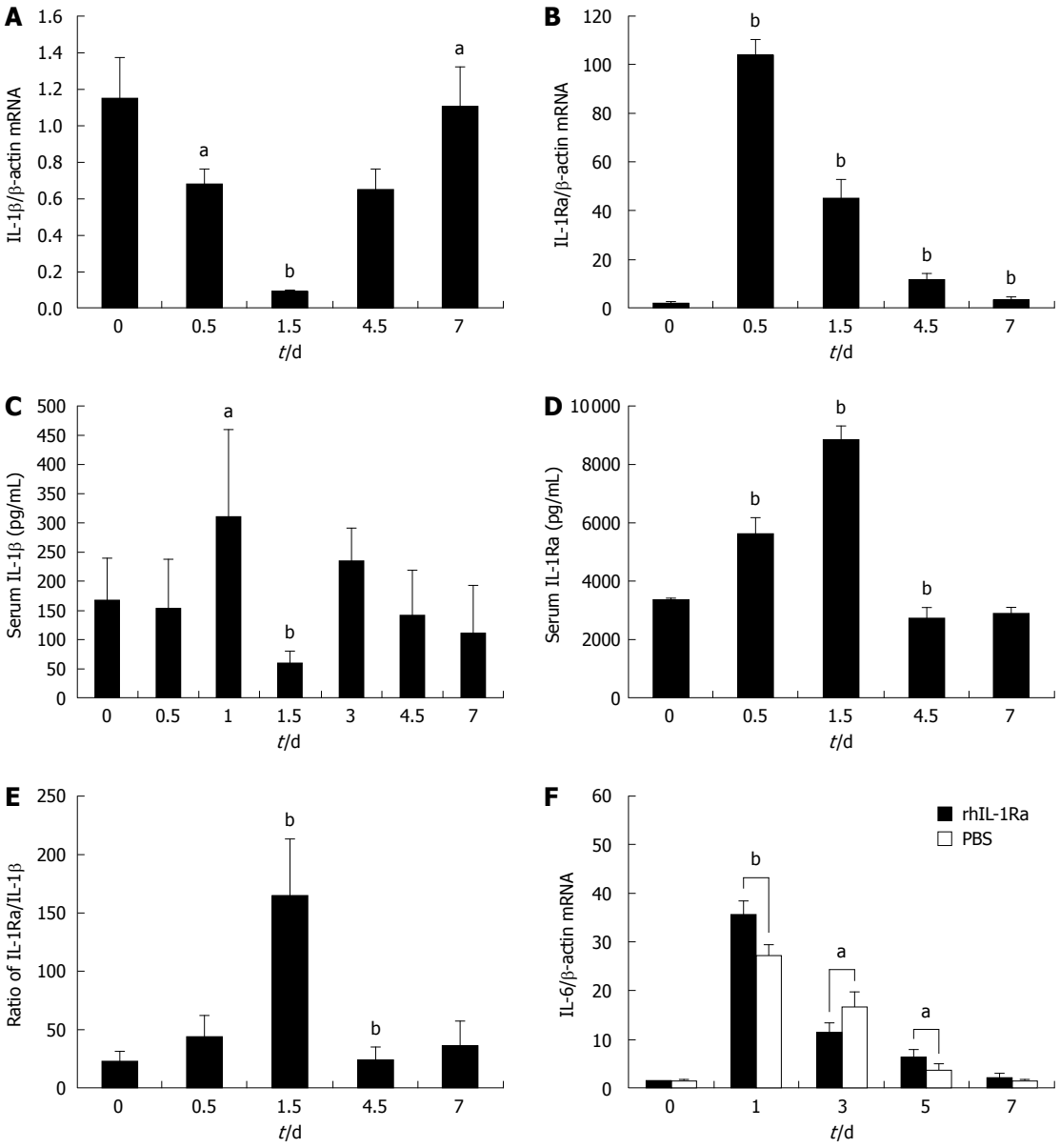


Figure 1 Interleukin (IL)-1β, interleukin 1 receptor antagonist (IL-1Ra) and IL-6 expression after carbon tetrachloride (CCl_4) (1 mL/kg) administration. A: Quantification of IL-1β mRNA levels; B: IL-1Ra mRNA levels; C: Serum IL-1β; D: Serum IL-1Ra; E: Ratio of serum level of IL-1Ra to IL-1β; F: Quantification of IL-6 mRNA levels of the livers treated with recombinant human IL-1Ra (rhIL-1Ra) or PBS. $^aP < 0.05$, $^bP < 0.01$.

flammation was dramatically decreased. We found the necrotic areas were significantly diminished around the central vein (Figure 2D and E) and centrilobular regions (Figure 2C) in rhIL-1Ra-treated mice at day 3. However, rhIL-1Ra did not cause any liver injury to healthy mice (Figure 2F). These findings indicate that rhIL-1Ra has a potent anti-hepatotoxic activity in reducing hepatocellular necrosis around the central vein.

rhIL-1Ra promotes hepatocyte proliferation from an early phase

We also investigated the proliferation of hepatocytes by immunostaining of PCNA in sections of liver tissue at days 2 and 3. Our PCNA staining confirmed that the number of positive cells increased sharply at day 2 (Figure 3C). Great numbers of hepatocytes (Figure 3E) could be detected in the liver sections of rhIL-1Ra-treated mice at day 3, which demonstrated that rhIL-1Ra significantly increased the number of $PCNA^+$ cells. In contrast, PBS-treated mice showed a much fewer number of $PCNA^+$ cells (Figure 3D and F). In our study, we also confirmed rhIL-1Ra was unable to induce hepatocyte proliferation (Figure 3A and B) in normal mice. Numbers of $PCNA^+$ cells (Figure 3G) in at least 12 mm^2 tissue sections were counted for each mouse, which showed that mice receiving rhIL-1Ra after CCl_4 injection gained the potent advantage of accelerating hepatocyte proliferation from an early phase.

rhIL-1Ra increases probability of survival after a lethal dose performance

In dose-response experiments, we found that 2.6 mL/kg

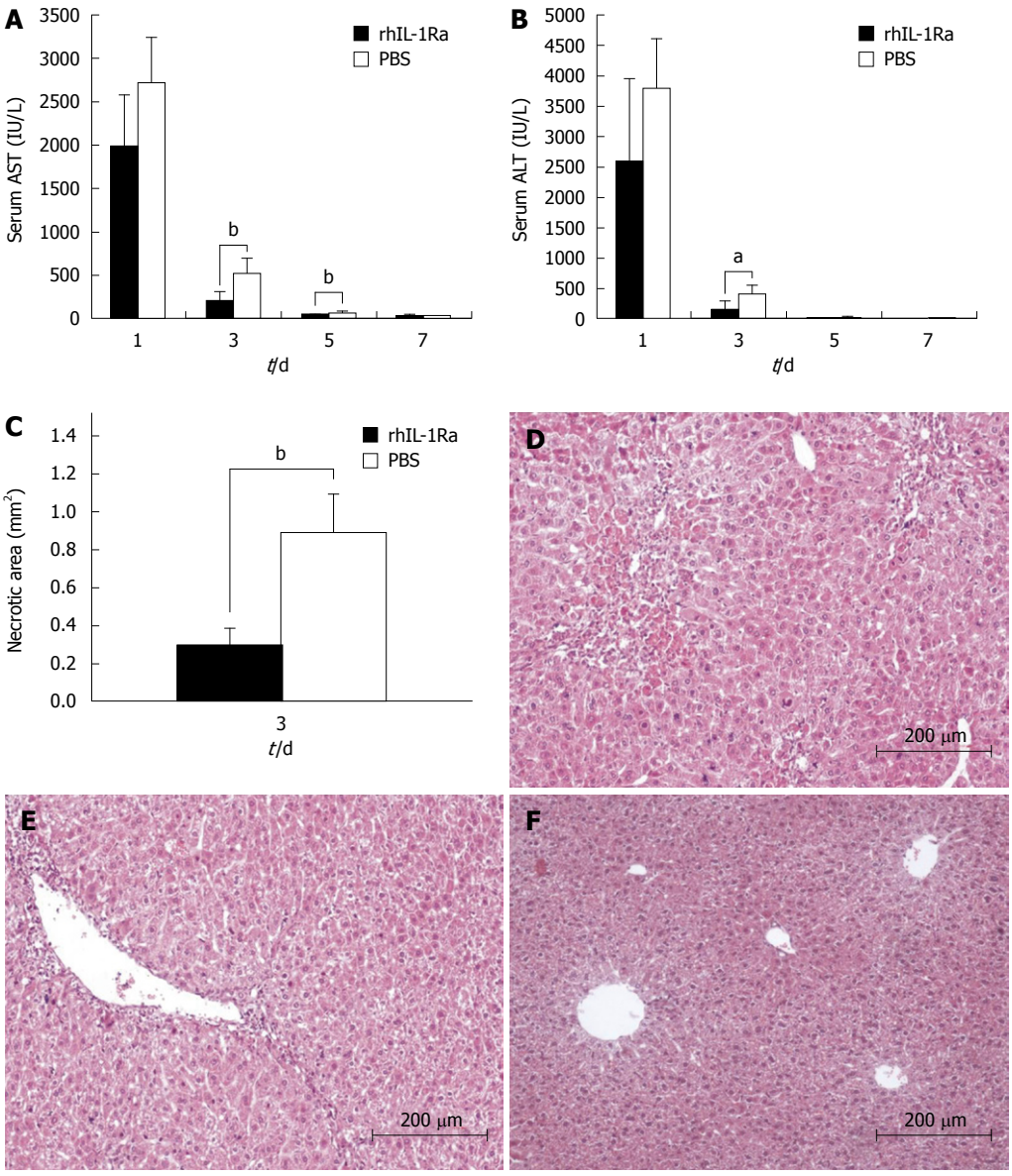
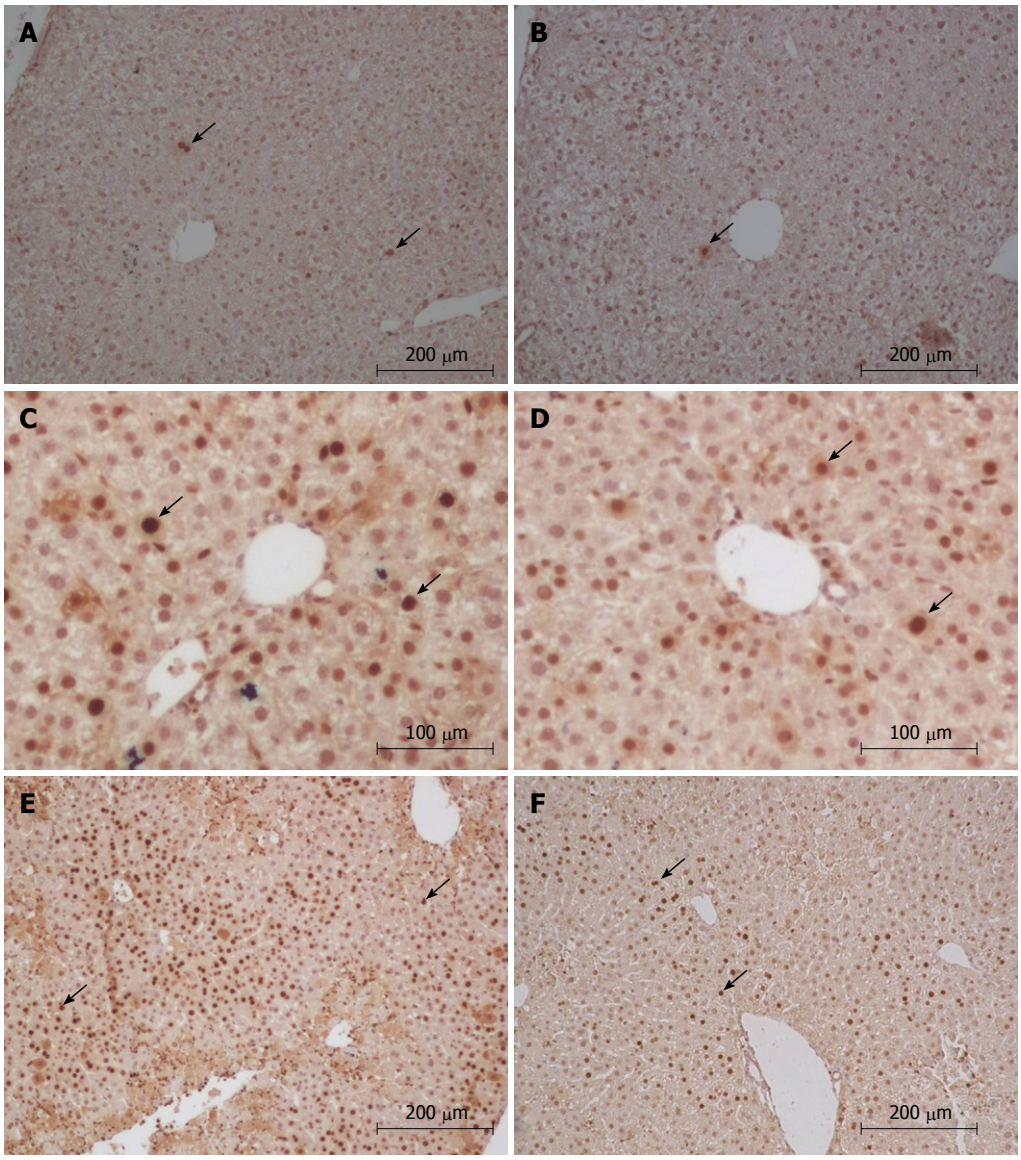


Figure 2 Acute liver injury (1 mL/kg CCl_4 administration) ± rhIL-1Ra. A: Serum aspartate aminotransferase (AST); B: Serum alanine amino transferase (ALT), with rhIL-1Ra or PBS; C: Necrotic areas. Representative findings from at least 12 mm^2 tissue sections were counted for each mouse; D-F: Hematoxylin and eosin (HE) stained liver sections; D: Group received PBS at day 3 after CCl_4 administration, shows necrosis with clusters of inflammatory cells around central vein (original magnification, × 100); E: Group received rhIL-1Ra at day 3 after CCl_4 administration, demonstrates mostly histological recovery with only inconspicuous necrosis still remaining around central vein and very few inflammatory cells are present (original magnification, × 100); F: Normal liver histology with rhIL-1Ra treatment, which shows no difference from normal liver tissue histology. $^aP < 0.05$, $^bP < 0.01$.

CCl_4 is a median lethal dose (mortality 50%, data not shown) within 24 h. rhIL-1Ra treatment in the CCl_4-induced acute liver failure model offers a survival benefit in treated mice, increasing the probability of survival significantly from 10.0% to 55.0% at day 3 after CCl_4 injection ($P = 0.006$, Figure 4).

DISCUSSION

The model of acute intoxication with CCl_4 has been used for decades to investigate the response of acute and chronic liver injury, because the elementary lesions caused by this hepatotoxin replicate those seen in most cases of human liver diseases. Pro-inflammatory cytokines such as IL-6 and IL-1β are believed to play a key role in the pathogenesis of CCl_4-induced liver injury[8,18,20,21], which make it a good model for us to study signal transduction and cell cycle events in a synchronized manner *in vivo*. The CCl_4-induced acute liver injury model is generated with 1 mL/kg dose for a typical hepatic injury, which would function as a strong regenerative stimulus. Regarding liver damage, IL-1Ra plays a critical role in the prevention of fatty liver and hypercholesterolemia under inflammatory conditions[22-24]. In this study, we investigated the severity of CCl_4-induced acute liver injury in mice after rhIL-1Ra treatment; the results demonstrate that rhIL-1Ra hypodermal injection affords protection from liver injury.



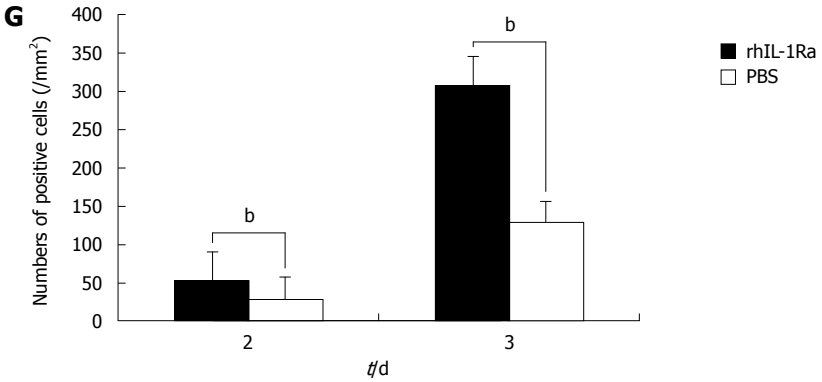


Figure 3 Immunostaining of proliferating cell nuclear antigen (PCNA) shows rhIL-1Ra promotes hepatocyte proliferation. A: Normal liver (original magnification, × 100); B: Normal liver in rhIL-1Ra treated mice (original magnification, × 100); C: Group received rhIL-1Ra at day 2 after CCl_4 administration, numerous $PCNA^+$ hepatocytes in centrilobular areas and scattered $PCNA^+$ hepatocytes at the edge of hepatocellular necrosis (original magnification, × 200); D: Group received PBS at day 2 after CCl_4 administration, few $PCNA^+$ hepatocytes in centrilobular areas at day 2 (original magnification, × 200); E: Group received rhIL-1Ra at day 3 after CCl_4 administration, numerous positive cells in centrilobular areas around central vein (original magnification, × 100); F: Group received PBS at day 3 after CCl_4 administration shows fewer numbers of positive cells (original magnification, × 100); G: Numbers of $PCNA^+$ cells after CCl_4 administration with rhIL-1Ra or PBS, at least 12 mm^2 tissue sections were counted for each mouse. Arrows point to PCNA+ hepatocytes. $^bP < 0.01$.

The balance between IL-1β and IL-1Ra has been extensively studied in a variety of experimental animal models of disease; either local overproduction of IL-1β or underproduction of IL-1Ra predisposes to the devel-

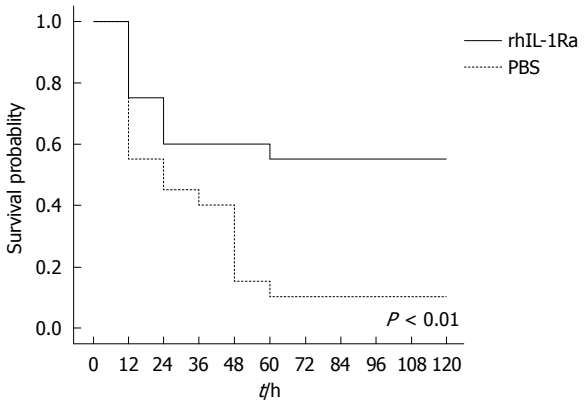


Figure 4 rhIL-IRa treatment increased probability of survival after a lethal dose administration of CCl_4 (2.6 mL/kg). Mice were administered with either PBS as a control (n = 20) or rhIL-1Ra (n = 20) twice a day for 5 d. Survivals were scored twice a day for 5 d, and the results were analyzed using the log-rank test and expressed as the Kaplan-Meier survival curves. P = 0.006.

opment of disease and the therapeutic administration of IL-1Ra is efficacious in preventing tissue damage[25]. Serum IL-1β and IL-1Ra are mediators of inflammation. Down-regulation in the release of the pro-inflammatory cytokine IL-1β and the up-regulation of its antagonist (IL-1Ra) may be a part of the inflammatory response to infection[8]. It is necessary to functionally inhibit the biologic effects of IL-1β on hepatocytes. We confirmed that liver IL-1β mRNA originally decreased in the first 12 h after CCl_4 treatment, and reached its lowest level at day 1.5. On the other hand, liver IL-1Ra mRNA increased to its highest level at day 1.5. These results show that the liver has a strong power to trigger compensatory growth mechanisms, which suppress the liver IL-1β expression. Several observations have emphasized that an adequate ratio of IL-1Ra to IL-1β is protective in inflammatory or immune liver disease[26-28]. Both serum IL-β and serum IL-1Ra were elevated after CCl_4 treatment, but serum IL-1Ra increased 100-fold greater than IL-1β at day 1.5. Serum IL-1Ra to IL-1β ratio was much higher in contrast to normal mice, which suggests a beneficial effect of IL-1Ra on CCl_4-induced acute liver injury. By analyzing the ELISA data of IL-1Ra and IL-1β, we found that serum IL-1β increased after CCl_4 treatment, which simultaneously stimulated the production of IL-1Ra as a compensatory reflection. The effects of IL-1Ra on blocking receptor binding of IL-1β during the acute-phase response may serve to suppress the inflammatory consequences of early IL-1β release after CCl_4-induced acute liver injury; with subsequent recovery, serum IL-1Ra decreased.

The level of serum aminotransferases is a very important marker to judge the severity of acute hepatic injury. After CCl_4 treatment the level of serum AST and ALT was significantly elevated, and attenuated by rhIL-1Ra in our experiment. The histological studies also showed that rhIL-1Ra inhibited inflammation and necrosis, which are the most common characteristics of CCl_4-induced liver damage. These findings suggest that rhIL-1Ra protects hepatocytes from the oxidative damage caused by CCl_4, which is likely due to inhibition of the pro-inflammatory mediators.

Serum IL-1Ra can rise dramatically during different inflammatory and non-inflammatory conditions such as sepsis[29] and chronic rheumatic diseases[30-32]. In addition, it plays a crucial role in regulating IL-1 signaling in various inflammatory states. IL-1Ra deficiency has been associated with major metabolic dysfunctions[33,34]. Serum levels of IL-1Ra were found to correlate with serum IL-6 concentrations[35], and administration of either IL-1 or IL-6 to patients increased the circulating levels of IL-1Ra[36,37]. Liver is a recognized target organ for pro-inflammatory cytokines such as TNFα, IL-1 and IL-6[38,39]. Regarding liver regeneration, IL-6 is thought to result in enhanced transcription, triggering hepatocytes to leave their quiescent state (G0) and enter a prereplicative phase (G1). Expression of IL-6 appears to be essential for the priming of hepatocytes[40,41]. Previous studies showed that IL-1Ra production was enhanced by IL-1β, and increasing IL-1β and IL-6 exhibits a strong stimulatory effect on the acceleration of IL-1Ra expression[8]. IL-6 is a marked signal to trigger liver regeneration, as demonstrated in a previous study[42], and in this current study, we found that the production of IL-6 could also be enhanced by excessive rhIL-1Ra treatment. *In vitro,* IL-1β inhibits hepatocyte proliferation[43]. Isoda *et al*[33] found that mRNA levels of IL-1β were significantly elevated in livers of IL-1Ra-/- mice, and liver growth is also inhibited by hepatocyte proliferation inhibitor and IL-1β[44,45]. It is of particular interest that excessive rhIL-1Ra inhibits the activity of IL-1β, which rapidly and significantly increased the number of $PCNA^+$ hepatocytes.

IL-1Ra is well tolerated clinically and has a short half-life, making it an ideal protective agent for acute hepatocellular damage and for accelerating liver regeneration. Healthy humans are the most sensitive indicators of IL-1 agonist activity: 1 ng/kg of intravenous IL-1β produces symptoms[46]. In contrast, the intravenous infusion of 10 mg/kg of IL-IRa in healthy humans, a 10 million-fold molar excess, gives no effect[47]. Histopathological studies showed that rhIL-1Ra-treated healthy mice show no changes in contrast with normal liver, and excessive rhIL-1Ra treatment could not induce hepatocellular proliferation in healthy mice. We used very classical methods to judge whether rhIL-1Ra has an effect on repairing the damage of mice CCl_4-induced acute liver injury and accelerating liver regeneration. The work indicates that rhIL-1Ra administration accelerates recovery from acute CCl_4-induced liver injury. To confirm that rhIL-1Ra dramatically prevented CCl_4-induced liver injury, a lethal dose of CCl_4 was used, and rhIL-1Ra treatment resulted in survival benefits in mice with acute hepatic failure. This work indicates that rhIL-1Ra accelerates recovery from acute CCl_4-induced liver injury and offers a strong survival advantage in injured mice.

Although additional studies are necessary to confirm this effect in humans, our findings provide a rationale

to develop new pharmacological strategies in the clinical management of patients with acute liver injury. This approach might also provide a novel therapeutic tool for regenerative liver cell therapy.

COMMENTS

Background

Interleukin 1 receptor antagonist (IL-1Ra) is a member of the interleukin 1 cytokine family and is a major anti-inflammatory cytokine. It acts as a natural inhibitor of the pro-inflammatory cytokines IL-1α and IL-1β through its action of blocking the binding of interleukin-1 to cell-surface receptors, and modulates a variety of IL-1-related immune and inflammatory responses. Although the anti-inflammatory effect of IL-1Ra has been described, the contribution of this cytokine in protecting from liver injury remains unclear.

Research frontiers

IL-1Ra is well tolerated clinically and has a short half-life, making it an ideal protective agent for acute hepatocellular damage and for accelerating liver regeneration. The authors used very classical methods to judge whether recombinant human IL-1Ra has effects on repairing the damage of carbon tetrachloride (CCl_4)-induced acute liver injury in mice and on accelerating liver regeneration.

Innovations and breakthroughs

The authors aimed to examine the effects of IL-1Ra as an acute phase protein in accelerating hepatocyte proliferation following CCl_4-induced liver injury. The results indicate that IL-1Ra is a critical factor that shows a potent antihepatotoxic activity in recovery of hepatocellular necrosis and accelerates liver regeneration during injury. IL-1Ra could provide a novel therapeutic approach by stimulating liver regeneration.

Applications

Although additional studies are necessary to confirm this effect in humans, the authors' findings provide a rationale to develop new pharmacological strategies in the clinical management of patients with acute liver injury. This approach might also provide a novel therapeutic tool for regenerative liver cell therapy.

Peer review

It is interesting and well-written. I suggest the authors perform electron microscopic examination in order to investigate the ultrastructural features of the hepatocytes of normal compared to recombinant human IL-1Ra-treated mice, and CCL_4-compared to recombinant human interleukin 1 receptor antagonist-treated mice.

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BRIEF ARTICLE

Anxiety and depression in adult patients with celiac disease on a gluten-free diet

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Abstract

AIM: To compare anxiety and depression levels in adult patients with celiac disease (CD) on a gluten-free diet (GFD) with controls.

METHODS: The levels of anxiety, depression and of a probable anxiety or depressive disorder were assessed by the Hospital Anxiety and Depression Scale in 441 adult patients with CD recruited by the German Celiac Society, in 235 age- and sex-matched patients with inflammatory bowel disease (IBD) in remission or with slight disease activity, and in 441 adult persons of a representative German general population sample (GP). Potential demographic (age, sex, social class, family status) and disease-related (latency to diagnosis, duration of GFD, compliance with GFD, thyroid disease) predictors of anxiety and depression in CD were tested for by regression analyses.

RESULTS: The level of anxiety in CD patients was predicted (R^2 = 0.07) by female gender (P = 0.01). Female sex (OR = 3.6, 95% CI: 1.3-9.4, P = 0.01) was associated with a probable anxiety disorder. Living alone (OR = 0.5, 95% CI: 0.2-0.9, P = 0.05) was associated with a reduced risk of an anxiety disorder. The level of depression and a probable depressive disorder were not predicted by any of the demographic and medical variables tested for. The levels of anxiety in patients with CD (6.6 ± 3.4) and with IBD (6.9 ± 3.7) were higher than those of persons in the GP (4.6 ± 3.3) (both P < 0.001). The levels of depression in persons with CD (4.2 ± 3.4), IBD (4.6 ± 3.4) and of the GP (4.2 ± 3.8) did not differ (P = 0.3). The prevalence of a probable anxiety disorder in persons with CD (16.8%) and IBD (14.0%) was higher than that of the GP (5.7%) (P < 0.001). The prevalence of a probable depressive disorder did not differ significantly between the three groups (P = 0.1).

CONCLUSION: Anxiety in adult German female celiacs on a GFD is higher than in persons of the GP. Female celiacs on a GFD should be screened for anxiety.

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Key words: Celiac disease; Anxiety; Depression; Gender

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INTRODUCTION

Celiac disease (CD) is an autoimmune disorder which is precipitated, in genetically predisposed persons, by the ingestion of gluten, the major storage protein of wheat and similar grains. Originally considered a rare malabsorption syndrome of childhood, CD is now recognized as a common condition that may be diagnosed at any age and that affects many organ systems. In most affected people, CD remains undiagnosed, although the rate of diagnosis is increasing. CD occurs in adults and children at rates approaching 1% of the population. The disease is recognized not only throughout Europe and in countries populated by persons of European ancestry but also in the Middle East, Asia, South America and North Africa[1]. The highest CD prevalence in the world is found in a North African population originally living in the Western Sahara with a figure of 5.6%[2]. Among adults, two to three times as many women have the disease as men, for unknown reasons. The therapy for the disease is a gluten-free diet (GFD); however, the response to therapy is poor in up to 30% of patients mainly because of non-adherence to the GFD[1].

Along with the many gastrointestinal, nutritional and metabolic consequences of CD, there have been significant concerns about increased rates of psychological symptoms and mental disorders in celiacs (CDs)[3]. Most studies have been on depression[4-17]. Case series in Gastroenterological departments[4] and case control studies in primary care demonstrated that depression can be a symptom of undiagnosed CD[5]. Whether the levels of depression in CDs under a GFD are comparable to those of controls [general population (GP), other chronic somatic diseases] is under debate. Some studies found more depression in CDs compared to controls of the GP[6-11,13,15,17], others did not[12,14,16]. A few studies also explored anxiety. Some studies reported more anxiety in CDs compared to controls[8,13], some did not[7,16]. The majority of studies were performed in Italy[6-11,13,17] and Scandinavia[12,15,16]. No study has been performed in Germany until now. Only two studies included more than 200 CDs[14,15].

The data regarding potential disease-related determinants of anxiety and depression in CDs on a GFD are also contradictory: one Italian study found no correlation between duration of, and compliance with, GFD and depressed mood[10], whereas another reported increasingly depressed mood with the duration of GFD[13]. An elevated risk of depression in CD patients with type 1 diabetes mellitus and autoimmune thyroiditis has been reported by one study for each of these diseases[14,9]. The influence of demographic factors which are associated with depression in the GP, such as marital status or social class index[18], were only controlled for in a minority of studies[7,8,13]. Moreover, gender aspects in anxiety/depression in CD have been considered only by a few studies, although Scandinavian studies found that female CDs reported more anxiety and depression than male patients[16]. These open questions on anxiety and depression in CDs on a GFD provided us with the aims of our study which were: (1) to explore the potential impact of disease-related, demographic and gender variables on anxiety/depression in CDs; and (2) to compare the levels of anxiety/depression and the frequency of anxiety and depressive disorder in CDs with a sample of the GP and with patients having another chronic somatic disease, with adequate large sample sizes, in a central European country (Germany).

MATERIALS AND METHODS

Ethics

The surveys were approved by the ethics committees of the University of Tübingen and Leipzig and of the board of physicians of the Saarland.

Patients

CD: Within the German Celiac Health Survey of the German Celiac Society DZG, a set of questionnaires was sent out in May 2005 by the DZG to 1000/18 355 of their members ≥ 18 years old, self-reporting a diagnosis of CD according to the membership directory of the DZG, following consecutive postal codes 0-9. To ensure a geographically representative sample every 18th member of the list was contacted by a letter signed by the board of directors of the DZG in which the aims of the study were explained. The letter included a set of questionnaires with a stamped self-addressed envelope enclosed. The patients were asked to give their written informed consent and to fill out and send back the questionnaires within four weeks. Patients < 18 years were not included in the study. There were no other primary exclusion criteria. All patients gave their voluntary informed consent after full written explanation of the aims of the study, including considerations regarding ethics and data protection and the anonymous deposition of the questionnaires[19].

Inflammatory bowel diseases: We selected inflammatory bowel diseases (IBDs) (patients with IBD) for comparison because both diseases have similar gastrointestinal (abdominal pain, diarrhea) and general (fatigue) symptoms and disease-associated disorders (e.g. bone, skin, liver).

Consecutive adult patients (≥ 18 years) with diagnosis of Crohn's disease or ulcerative colitis confirmed

by endoscopy and histology, attending three tertiary care centers for evaluation and/or therapy and members of the German's Crohn's Disease/Ulcerative Colitis Foundation (DCCV), were invited to participate in a study on health-related quality of life (HRQOL)[20]. Patients unable to speak German or with suspicious diagnosis and indeterminate colitis were excluded. Based on the hospital registry and medical documentation, a total of 730 adults were identified who had been attending the specialized outpatient clinic of the Tübingen University Hospital at least once in the last three years with confirmed diagnosis of either Crohn's disease or ulcerative colitis. Every one of these patients received a set of questionnaires by mail and was asked to return it once completed. One reminder was sent out three weeks after initial postal contact. Forty-five consecutive IBD patients of the coloproctological outpatient department of the Department of General and Abdominal Surgery of the University of the Saarland and 23 consecutive IBD inpatients of the Department of Internal Medicine I of the Klinikum Saarbrücken were asked to participate in the study during regular outpatient visits or during a hospital stay after admission for any acute complications of their disease. Physicians were trained to offer instruction and help if persons did not understand the meaning of questions when needed, to collect the questionnaires and to record clinical data using standardized forms. The study took place between January and June, 2000. In edition 4 (2000) of the journal of the German Crohn's disease/Ulcerative Colitis Foundation (DCCV) "Bauchredner", the main topic was "Health-related quality of life and life satisfaction in IBD" and all readers suffering from IBD were invited to participate in our study. The DCCV is the largest patient association for IBD in Germany with around 15 000 members (patients, relatives, physicians). Seventy-six patients indicated their interest to the DCCV and received the set of questionnaires by mail and were asked to return the completed set to the study center in Saarbrücken.

Disease activity was measured by the German Inflammatory Bowel Disease activity Index (GIBDI$_{\text{Crohn's disease}}$ resp. GIBDI$_{\text{Ulcerative colitis}}$). The GIBDI had been validated for the assessment of disease activity in surveys within the German Competence Network Inflammatory Bowel Diseases[21].

General German population: A representative sample of the German GP was selected with the assistance of a demographic consulting company (USUMA, Berlin, Germany). The random selection was based on multistage sampling with three stages (according to the typical random selection procedure in national surveys in Germany). First, 258 sample point regions were randomly drawn from the last political election register, covering rural and urban areas from all regions in Germany. The second stage was a random selection of households using the random route procedure (based on a starting address). The third stage was a random selection of household respondents with the Kish selection grid. The sample aimed to be representative in terms of age, gender, and education for the general German population. The inclusion criteria for the study were age at or above 14 and the ability to read and understand the German language. All subjects were visited by a study assistant informed about the investigation. Self-rating questionnaires were presented. The assistant waited until participants answered all questionnaires, and offered help if persons did not understand the meaning of questions. Data collection took place in 1998[22].

Questionnaires

Medical questionnaires: In both patient surveys a self-developed medical questionnaire assessing the history of the disease and therapy, current symptoms necessary to calculate activity scores, disease-related comorbidities and current medication was included. CDs were asked about comorbid dermatitis herpetiformis, diabetes mellitus type 1, autoimmune thyroiditis, autoimmune liver-biliary tract disease, recurrent aphtae, anemia, Addison's disease, osteoporosis and duodenal carcinoma or lymphoma. Latency of diagnosis was calculated by the reported difference of the time between first medical assessment because of CD-associated symptoms and final diagnosis.

The demographic questionnaire of the German Competence Network "Inflammatory Bowel Diseases"[21] was included in both patient surveys. By questions on education, the occupational status and the available income were used to calculate a social index. Data of this social index can be compared to a representative sample of the general German population[23].

The Hospital Anxiety and Depression Scale (HADS) was specifically designed for the assessment of anxiety and depression in patients with physical illness. With 7 items each on a 4-point Likert scale ranging from 0 to 3, subscale scores for anxiety and depression can be calculated. Scores $\geqslant$ 11 on the anxiety scale are indicative of a probable anxiety disorder (mainly general anxiety disorder). Scores $\geqslant$ 11 on the depression scale are indicative for a probable depressive disorder[24,25]. The HADS is a reliable and valid psychological screening tool for anxiety and depressive disorder in physically ill persons[25] with a validated German version available[26].

Statistical analysis

All statistical analyses were performed with the Statistical Package for the Social Sciences (SPSS, Release 17.0, Chicago 2008). One missing item of each subscale of the HADS was substituted by the individual median. If more items were missing the patient was excluded from analysis.

Data derived from descriptive statistical analysis are presented in the form of percentages for categorical variables and of the mean and the standard deviation (SD) for continuous data. Categorical data were compared using the χ^2-test and continuous data by *t*-tests or analysis of variance with post-hoc Bonferroni adjusted pairwise comparisons. In addition, anxiety and depres-

sion scores were compared between CDs and IBDs using analysis of covariance adjusting social class index.

Because we were interested in assessing the possible impact of demographic and medical factors on anxiety and depression, the anxiety and depression scores of the HADS served as dependent variables for a multiple regression analysis. Present age, family status, gender, social class index, latency of diagnosis, duration of and compliance with GFD and CD-associated thyroid disease were included as independent variables in a direct multiple regression analysis.

In order to estimate the risk for a possible anxiety and depressive disorder (coded 1 = no, 2 = yes), a logistic regression (all independent variables were included within a single step; no automated stepwise selection procedures were used) was performed with the same variables. The variables were coded as follows for the regression analyses: (1) Demographic variables: sex (1 = male, 2 = female), present age (continuous), living in a family or partnership (1 = yes; 2 = no) and social class index (1 = low, 2 = middle, 3 = high); and (2) CD-associated variables: reported latency between first medical assessments because of CD-associated symptoms and final CD diagnosis (continuous), reported duration of GFD (continuous), reported adherence to GFD (1 = rarely; 2 = sometimes; 3 = most of the time; 4 = all of the time), thyroid disease (1 = no, 2 = yes), DM type 1 (1 = no, 2 = yes).

The internal validity of the model of the logistic regression analysis was tested by the omnibus test of the model coefficient and the Hosmer-Lemeshow-test.

A prevalence of mental disorders of 10% estimated by the HADS in the general German population[23] given a sample size of 219 for each group was calculated to provide 80% power to assess a 10% difference between CD and controls.

Due to multiple comparisons the level of significance was set to $P = 0.01$ for group comparisons.

RESULTS

Return rates

CD: Almost half of the total questionnaires (52.2%, 522 of 1000) were returned. Since six questionnaires were excluded due to missing data, a total of 516 questionnaires were usable for further analyses. Of these, 213 (41.3%) of the respondents indicated that the CD diagnosis was made by duodenal biopsy, 37 (7.2%) by serological tests (CD-specific antibodies), 34 (6.6%) by stool tests (transglutaminase antibodies), and 232 (45.0%) by duodenal biopsy plus serological tests. The 445 patients reporting a biopsy-proven CD were included for further analysis. HADS data were available from 441 patients. Patients who took part in the survey did not differ from those who did not send back the questionnaires in terms of age, sex and geographical region of Germany.

IBD: The total response rate was 63.4% (550/868). Of the 550 returned questionnaires, 128 were excluded from analysis due to colo- or ileostoma ($n = 59$), uncertain diagnosis ($n = 16$), impossibility of calculation of GIBDI because of missing data ($n = 51$), missing HADS data ($n = 6$) or missing declaration of consent ($n = 2$). Patients with moderate ($n = 40$) and severe ($n = 10$) disease activity were excluded from comparison. The IBD group thus consisted of 366 patients; 50% were females. To ensure a sex-matched comparison with CDs, 131 randomly selected male patients were excluded from analysis. Of the 235 included for comparison, 138 (56.3%) patients were in remission, 107 (43.7%) patients had a slight disease activity.

To check for a possible selection bias, the data of responders were compared to the known structural data on all outpatients of the Tübingen outpatient clinic (place of residence, diagnosis, gender, age, duration of disease, age at disease onset, number of consultations in 2000). No indication of systematic selection effect was found in respondents *vs* non-respondents. Likewise, there was no difference in terms of gender and age in patients from the departments of the Saarland University Clinics who did and did not participate. There were no significant differences in the demographic variables and HADS scores of the patients included in and excluded from comparison.

GP: The initial sample consisted of 2952 subjects of whom 2037 (69.0%, $n = 1142$ women) fully participated. Forty-five subjects < 18 years were excluded. Thus the GP sample consisted of 1992 adult persons. Of these, 441 GPs (persons of GP) (346 women, 95 men) were randomly selected for comparison. There were no significant differences in the demographic variables and HADS scores of the persons included in and excluded from comparison.

Medical and demographic data of the celiac sample

The sample consisted of 78.5% women. The most frequently self-reported CD-associated diseases were dermatitis herpetiformis Duhring ($n = 45$, 9.3%) and autoimmune thyroiditis ($n = 25$, 5.6%). Type 1 diabetes mellitus was reported by 2 patients (0.5%). There were no gender differences in the demographic and medical variables assessed. The level of anxiety ($P = 0.0002$) and the frequency of a probable anxiety disorder ($P = 0.01$) were higher in female than in male CDs (Table 1).

Predictors of anxiety and depression in CD

Anxiety was significantly predicted only by female gender ($R^2 = 0.07$, $P = 0.01$). Depression was not significantly predicted by any of the medical and sociodemographic variables tested.

Predictors of a probable anxiety and depressive disorder

Because only in 2/8 patients with CD and diabetes mellitus type 1 an anxiety or depressive disorder was diagnosed, this variable was not entered into the regression analysis.

Anxiety disorder: The model correctly classified 84.8%

Table 1 Demographic data of the celiac disease sample with gender comparison

Variable	Total sample	Females	Males	Test-value *P*-value
Sex (*n*, %)	441 (100)	346 (78.5)	95 (21.5)	
Age, mean (SD)	46.3 (15.1)	45.7 (15.0)	48.5 (15.0)	Not significant
Living in partnership (*n*, %)	334 (76.1)	253 (73.8)	80 (84.2)	Not significant
Social class index (*n*, %)				Not significant
Lower class	26 (6.3)	18 (5.6)	7 (7.5)	
Middle class	271 (65.3)	220 (68.8)	50 (53.8)	
Upper class	118 (28.4)	82 (25.6)	36 (38.7)	
Number of CD-associated diseases (*n*, %)				Not significant
None	240 (55.2)	175 (51.3)	65 (69.9)	
One	118 (27.1)	100 (29.3)	17 (18.3)	
Two	48 (11.0)	40 (11.7)	8 (8.6)	
> Two	29 (6.7)	26 (7.7)	3 (3.2)	
Latency to diagnosis, yr, mean (SD)	5.6 (10.0)	5.9 (9.8)	4.9 (10.7)	Not significant
Years since start of GFD, mean (SD)	8.5 (7.7)	8.5 (7.7)	8.7 (7.7)	Not significant
Compliance with GFD				Not significant
Rarely		20 (5.8)	2 (2.1)	
Sometimes		9 (2.9)	0	
Most of the time		86 (25.0)	30 (32.3)	
All of the time		229 (66.6)	61 (65.6)	
Anxiety, mean (SD)	6.7 (4.09)	7.1 (4.1)	5.3 (3.5)	t = -3.7, P < 0.001
Depression, mean (SD)	4.1 (3.6)	4.3 (3.8)	3.2 (3.9)	t = -2.5, P = 0.01
Probable anxiety disorder (*n*, %)	68 (15.4)	63 (18.2)	5 (5.2)	χ^2 = 9.6, P = 0.001
Probable depressive disorder (*n*, %)	25 (5.7)	24 (6.9)	1 (1.0)	Not significant

Some discrepancies between total number of patients and absolute numbers of some variables are due to missing data. CD: Celiac disease; GFD: Gluten-free diet.

of the patients. Only female sex (OR = 3.6, 95% CI: 1.3-9.4, P = 0.001) was associated with a probable anxiety disorder. Living alone (OR = 0.45, 95% CI: 0.20-0.99, P = 0.05) was associated with a reduced risk of a probable anxiety disorder. The omnibus test of the model coefficient was significant (χ^2 = 20.2, P = 0.009). The level of significance in the Hosmer Lemeshow test was P = 0.5 (χ^2 = 6.9) above the predefined P-value of 0.05, thus confirming the adequacy of the model.

Depressive disorder: None of the variables tested was significantly associated with a probable depressive disorder. The omnibus test of the model coefficient was not significant (χ^2 = 13.5, P = 0.10).

Comparison with GP and patients with chronic IBD

In the CD sample, there were more patients of a higher social class than in the IBD sample (χ^2 = 14.0, P = 0.006). There were no differences between the samples in the other variables tested (Table 2).

The level of anxiety in CDs (6.7 ± 4.0) and IBDs (6.9 ± 3.7) was higher than that seen in GPs (4.6 ± 3.6) (all P < 0.001). After adjusting for social class there was no significant difference in the anxiety level between CDs and IBDs (F = 1.3, P = 0.3).

The level of depression in CDs (4.1 ± 3.6), IBDs (4.6 ± 3.4) and GPs (4.2 ± 3.8) did not differ (P = 0.3). After adjusting social class index, IBDs reported higher depression than CDs (F = 3.9, P = 0.02).

The frequency of a probable anxiety disorder was higher in CDs (15.4%) and IBDs (14.0%) than in GPs (5.7%) (all P < 0.001). There was no difference in the frequency of a probable depressive disorder between the three groups (P = 0.1).

DISCUSSION

We assessed medical and demographic predictors of anxiety and depression in adult patients with CD and compared their levels of anxiety and depression and the frequency of an anxiety or depressive disorder with age- and sex-matched patients with IBD in remission or slight disease activity, and with a representative German population sample. Only female sex, but not medical variables such as duration of and compliance with GFD, predicted anxiety. Depression was not predicted by any of the medical and demographic variables. Anxiety, but not depression, was higher in CDs and IBDs than in GPs.

Comparisons of the results with other studies

Factors associated with anxiety and depression in CD: Two Italian studies found no gender differences in anxiety and depression[10,27] whereas two Scandinavian studies reported lower levels of psychological well-being in female CD patients[16,28]. The higher levels of anxiety in German and Scandinavian women might be explained by general gender differences and/or CD-specific factors. Higher levels of anxiety and frequency of anxiety disorder in women have also been found in the general German population[22]. A higher psychosocial burden on female CDs because of buying and preparing food for the family can be presumed[28,29].

Table 2 Comparisons of patients with celiac disease with patients with inflammatory bowel disease in remission or having slight disease activity and with subjects of the general population

Variable	Celiac disease *n* = 441 (1)	Inflammatory bowel disease *n* = 235 (2)	General population *n* = 441 (3)	Overall comparison	Comparison subgroups
Sex (female) (*n*, %)	346 (78.5)	183 (77.9)	346 (78.5)	Not significant	
Age (mean, SD)	46.3 (15.1)	44.9 (12.4)	49.9 (16.8)	Not significant	
Social class index (*n*, %)			No comparable data available	Not possible	$\chi^2 = 14.5$
Lower	26 (6.3)	28 (11.9)			$P < 0.01$
Middle	271 (65.3)	167 (71.1)			
Upper	118 (28.4)	40 (17.0)			
Living in partnership or with family (*n*, %)	334 (76.1)	187 (80.3)	No comparable data available	Not significant	
Anxiety				$F = 45.9$	1, 2 > 3
Total, mean (SD)	6.7 (4.0)	6.9 (3.7)	4.6 (3.3)	$P < 0.001$	
Depression				Not significant	
Total, mean (SD)	4.1 (3.6)	4.6 (3.4)	4.2 (3.8)		
Probable anxiety disorder				$\chi^2 = 23.2$	1, 2 > 3
Total (*n*, %)	68 (15.4)	33 (14.0)	25 (5.7)	$P < 0.001$	
Probable depressive disorder				Not significant	
Total (*n*, %)	25 (5.7)	12 (5.1)	38 (8.6)		

In contrast to findings in the GP[18,30], low social class index was not associated with anxiety and depression in CDs. In contrast to the GP[18,30], living in a family was associated in CDs with the risk of an anxiety disorder. We speculate that the problems of buying and preparing food in a family with CDs and non-CDs might lead to financial and interpersonal problems which can contribute to an anxiety disorder.

In line with one Italian longitudinal study[7] and one Italian cross-sectional study[10], we found no association between depressed mood and the duration of GFD. We also found no association between anxiety and the duration of GFD. This finding is not contradictory to the results of longitudinal studies which reported a decrease of anxiety (state) after starting a GFD[7,12]: a GFD may reduce psychological symptoms due to malabsorption of tryptophan, but may also increase psychological distress associated with the financial and social restrictions of GFD and disease-related worries[6,31].

We confirm the results of two Italian studies which found no association between non-compliance with GFD and anxiety/depression[10,13].

The limited number of CDs with diabetes mellitus type 1 prohibited a comparison with an American study which reported a higher prevalence of depression in CD patients with type 1 diabetes mellitus (5.8% of the sample) compared to celiacs without type 1 diabetes mellitus[14]. We could not confirm a higher prevalence of depressive disorders in CDs with autoimmune thyroiditis[9].

Anxiety/depression in CDs compared to controls: The different instruments used to assess anxiety and depression do not allow a comparison of prevalence rates in studies on anxiety/depression in CD. Moreover, the studies differed in the ways of recruiting patients and in the type of controls used. Some studies used healthy subjects[6-10,13,14,17], GP[15,16] or patients with diabetes[13], IBD[6], irritable bowel syndrome[14] and chronic hepatitis[10] for controls. Despite these limitations, we can state that our finding that CDs are not more depressed than controls is in line with the results of one American[14] and two Scandinavian[11,16] studies and in contrast to the findings of the Italian studies[6-11,13,17]. Our finding of more anxiety in CDs compared to GP controls is in line with that of Italian studies[7-9] and in contrast to Scandinavian studies[11,16].

Limitations of the study

Some limitations of the study have to be considered. First, the study population consisted of adult CDs recruited among members of the German Celiac Society which may have induced a selection bias. A large hospital-based register of CD patients is not available in Germany. Furthermore, to our knowledge, most CDs attending tertiary care outpatient departments in Germany are associated with the German Celiac Society. Additionally, we cannot exclude a response bias of CDs sending back the questionnaires. Nevertheless, the CD sample of our study is the best available one in Germany. Second, we cannot exclude a response bias of CD and IBD patients sending back the questionnaires. Third, another potential bias of the study is the different approach to completing and returning the questionnaires. In the subsamples of the GP and the IBD patients of the departments of Homburg/Saar and Saarbrücken, help was offered if persons did not understand the meaning of questions and the questionnaires were recollected by research assistants or physicians. The questionnaires of the celiac and IBD sample of the department of Tübingen were completed without potential external support and returned by mail. Fourth, all data on the history and type of diagnosis, CD-associated diseases and on adherence to regimen relied solely on the participants' self report. Due to data protection in this type of survey, information from clinical records, interviews or serological/radiological tests was not available. Fifth, due to the type of study (postal survey) we were unable to use standardized psychiatric interviews for the confirmation of a probable

mental disorder when the critical cut-off scores of the HADS were surpassed. Furthermore, the items on anxiety of the HADS cover general anxiety but not phobic symptoms. Therefore, our data cannot comment on the high frequency of social phobia seen in Italian patients[8]. However, the HADS is one of the best screening tools available for anxiety and depressive disorders in patients with somatic diseases[25]. Finally, because of the cross-sectional nature of the study the results show only associations between factors and do not allow for conclusions for causality.

In conclusion, German patients with CD on a GFD do not differ from the GP in levels of depression, but there is a subgroup of mainly female CDs with a probable anxiety disorder. Mental disorders increase the risk of a reduced health-related quality of life[31] and of irritable bowel-like symptoms in CDs[32]. A screening for comorbid anxiety disorders in female patients with CD on a GFD by general practitioners and gastroenterologists is therefore recommended, and screening is possible by using the ultra-brief screening scale for anxiety and depression, PHQ-4[33]. The two questions on anxiety in the PHQ-4 can be directly asked: "Over the last two weeks, have you often been bothered by feeling nervous, anxious, or on edge? Over the last two weeks, have you often not been able to stop or control worrying?"

COMMENTS

Background

Previous studies have yielded conflicting results regarding the presence of an association of celiac disease (CD) in patients on a gluten-free diet (GFD) and anxiety/depression.

Research frontiers

Anxiety and depression are common symptoms in the general population (GP) as well as in other chronic somatic diseases and are associated with demographic factors such as age, gender and social class. Most studies on anxiety/depression in CD were not adequately powered to detect differences in anxiety and depression between CD and controls. In this study with adequately powered sample sizes, the authors demonstrate that anxiety in CD patients on a GFD is higher than in the GP and comparable to that of patients with inflammatory bowel disease who are in remission and have slight disease activity.

Innovation and breakthroughs

Recent reports have highlighted the importance of female gender and other autoimmune disorders associated with CD as predictors of depression in CD. This is the first study to report an association between anxiety disorder and female gender in CD. The study did not confirm findings that autoimmune thyroid disorder is a risk factor for depressive disorder in CD.

Applications

The study substantiated the necessity of exploring not only somatic but also anxiety symptoms in female CD patients on a GFD.

Peer review

This paper is worthy of publication as the first comprehensive study of anxiety and depression in German adult CD. It needs to be substantially revised, reduced in size, particularly in the Discussion and number of tables, and be less speculative.

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BRIEF ARTICLE

Segmental duodenectomy for gastrointestinal stromal tumor of the duodenum

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Abstract

AIM: To evaluate the results of segmental duodenectomy (SD) and pancreaticoduodenectomy (PD) for duodenal gastrointestinal stromal tumor (GIST) and help clinicians with surgical management.

METHODS: All patients who underwent surgery for non-metastatic GIST of the duodenum in a single institution since 2000 were prospectively followed up. Seven patients (median age 51 years, range: 41-73 years) were enrolled: five underwent SD and two underwent PD.

RESULTS: All the patients had a complete resection (R0), with no postoperative morbidity and mortality. Among the SD group, GIST was classified as low risk in two patients, intermediate risk in two, and high risk in one, according to the Fletcher scale, (*vs* two high risk patients in the PD group). With a median follow-up of 41 (18-85) mo, disease-free survival (DFS) rates were 100% after SD and 0% after PD ($P < 0.05$). The median DFS was 13 mo in the PD group.

CONCLUSION: Whenever associated with clear surgical margins, SD is a reliable and curative option for most duodenal GISTs, and is compatible with long-term DFS.

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Key words: Gastrointestinal stromal tumor; Duodenal neoplasms; Segmental duodenectomy; Pancreaticoduodenectomy

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INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of the digestive tract, with an estimated annual incidence between 10 and 20/10^6 people[1,2]. Although GISTs are encountered all along the digestive tract, the most frequent sites of occurrence are the stomach (50%) and small bowel (30%). Duodenal GISTs are less frequent and account for < 5% of cases, but still represent approximately 30% of primary duodenal tumors[3].

Surgery is still the only curative approach for GIST[4-6], but the optimal surgical procedure for duodenal GIST remains to be established. A number of authors have reported various procedures including pancreaticoduo-

denectomy (PD), pancreas-sparing duodenectomy, segmental duodenectomy (SD), or wedge local resection, but few have correlated the different options with oncological results[7-16].

Two major tumor characteristics have to be considered for surgical resection of duodenal GIST, which differs from duodenal adenocarcinoma[17]. First, GIST spreads specifically hematogenously and is rarely, if ever, associated with lymphatic invasion, as in other sarcomas[18]. Secondly, GISTs are well encapsulated tumors that rarely have a tendency to local invasion[4]. For these reasons, radical lymphadenectomy or extended resection of adjacent organs should not confer a survival advantage in non-metastatic duodenal GIST[6,19,20].

Therefore, this study was undertaken to audit the oncological results of segmental duodenal resection in comparison with more extensive procedure such as PD for primary non-metastatic duodenal GIST.

MATERIALS AND METHODS

This was a prospective cohort study of all surgical patients treated in our department for primary non-metastatic duodenal GIST from 2000 to 2008. Inclusion criteria were patients presenting with suspicion of non-metastatic duodenal GIST in a single hospital (Figures 1 and 2). Exclusion criteria were a clearly metastatic disease, poor health condition that precluded laparotomy (severe pulmonary disease, non-treatable coagulation abnormality), and patient's refusal to participate in this study. All patients had complete surgical resection (R0). Seven cases were included. Median age was 51 years (range: 41-73 years).

Five patients had an SD and two a cephalic PD. PD was the operation chosen for relatively large and difficult-to-reach tumors (first and second part of the duodenum). Among the SD group, one patient had a duodenal patch resection of a small D1 GIST with direct closure (case 3, Table 1), the others had a complete SD with latero-lateral duodeno-jejunal reconstruction (Figure 3).

Pathological diagnosis of GIST was confirmed for all according to histological and immunohistochemical work-up. All tumors were c-kit positive. GIST was classified according to the Fletcher scale[21] and our scale[4].

The primary endpoint for this analysis was disease-free survival (DFS), which was defined as time from surgery to GIST recurrence. Follow-up was available for all patients at the date set for collecting data, November 2008. Follow-up was carried out through routine visits at our Outpatient Oncological Clinic. Clinical assessment was made every 3 mo during the first 2 years after surgery and every 6 mo thereafter, with detection of recurrence as soon as possible to allow adjuvant therapy with imatinib. Yearly chest X-rays and abdominal computed tomography (CT) were routinely performed in all patients and additional imaging was requested when clinical suspicion of GIST recurrence occurred. The median follow-up was 41 (18-85) mo.

Statistical analysis

Statistical analysis was performed using GraphPad InStat (GraphPad Software, San Diego, CA, USA). When appropriate, data were analyzed using two-sided Fischer's test or two-sided t test. $P < 0.05$ was considered statistically significant.

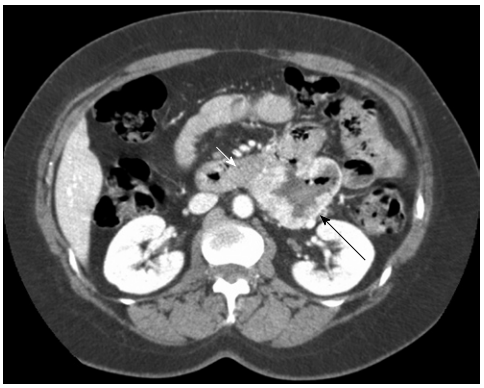


Figure 1 Gastrointestinal stromal tumor (GIST) located in the third part of the duodenum with typical computed tomography (CT) appearance. Note the typical CT appearance of GIST with an area of necrosis (central cavitations with surrounding highly vascular tissue) (white arrow: pancreas; black arrow: GIST).

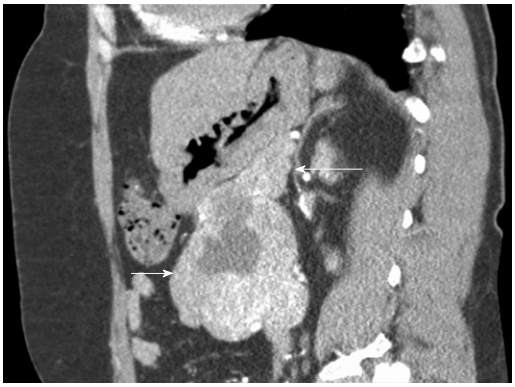


Figure 2 GIST located ion the horizontal duodenum (patient 5, Table 1). Note the close relation between the GIST (short arrow) and the pancreas (long arrow), which was easily dissected during surgery.



Figure 3 Duodeno-jejunal anastomosis. Latero-lateral duodeno-jejunal anastomosis between the second part of the duodenum and the first jejunal loop was easily performed after distal duodenectomy and Kocher maneuver.

RESULTS

Study population and pathological data

Seven patients were included in the analysis. Four patients presented with upper digestive tract hemorrhage,

Table 1 Patients characteristics and follow-up

Cases	Localization	Size (cm)	Mitosis/50 HPF	Fletcher grade	Surgery	Follow-up	DFS	Second-line therapy
1	D2	6.5	34	HR	PD	60	13	Imatinib and hepatectomy
2	D1	7	19	HR	PD	56	12	Imatinib
3	D1	2.5	3	LR	SD	85	85	-
4	D2	2	2	LR	Atypical duodenectomy	40	40	-
5	D2-3	10	10	HR	SD	37	37	-
6	D3	7	2	IR	SD	29	29	-
7	D4	5.5	5	IR	SD	18	18	-

D1: First part of the duodenum; D2: Second part of the duodenum; D3: Third part of the duodenum; D4: Forth part of the duodenum; HR: High risk; IR: Intermediate risk; LR: Low risk GIST according to Fletcher scale; HPF: High-power field; DFS: Disease free survival.

the others with abdominal discomfort. In all patients, the diagnosis of duodenal GIST was made through CT after it was suspected by endoscopy in those with digestive bleeding. No preoperative biopsies were performed.

All GISTs were localized without metastases or peritoneal dissemination. According to the Fletcher classification, the GISTs were considered as high risk in the two patients in the PD group, and in the SD group as high risk in one, intermediate risk in two, and low risk in two patients ($P > 0.05$). According to our classification, the two GISTs in the PD were classified as malignant, as was one of the five GISTs in the SD group ($P < 0.05$).

Postoperative outcomes

No postoperative morbidity and mortality were recorded. All patients had complete surgical (R0) resection of their duodenal GIST. Five patients underwent SD and two PD (Table 1). GIST-free surgical margins along the duodenum ranged from 0.5 cm to 3 cm.

Follow-up results

The median follow-up was 41 mo (range: 18-85 mo). Median follow-up was 58 mo (range: 56-60 mo) and 37 mo (range: 18-85 mo) for the PD and SD group, respectively. Two patients in the PD group demonstrated recurrence with a median disease-free interval of 13 mo, whereas no recurrence was observed in the SD group ($P < 0.05$). All patients are alive and disease free in the SD group with a median DFS of 37 mo. The two patients with recurrence after PD presented with liver metastases, which were treated with imatinib mesylate, and one was also treated with partial hepatectomy. A statistically significant difference was detected between the PD and SD group for DFS ($P = 0.048$), however, this should be balanced by a higher rate of malignant GIST and longer median follow-up in the PD group.

DISCUSSION

The optimal surgical procedure for GISTs of the duodenum remains poorly defined in terms of oncological results. This study was undertaken to compare oncological results of SD and the more radical PD. According to our data, duodenal GIST prognosis is dependent on tumor malignant potential when clear surgical margins can be achieved and not on size of surgical margins or lymphatic dissection. The data presented herein demonstrate that SD is associated with prolonged DFS.

Surgical resection is still the only curative therapy for GIST[1,6,19]. GISTs are known to be resistant to chemotherapy and radiotherapy, and the recently developed molecular targeted therapies (imatinib mesylate and sunitinib), while being highly effective in disease control, are not curative[1,5,6]. The optimal surgical procedure for duodenal GIST remains poorly defined in terms of oncologic results. The reports in the literature addressing surgical procedures for duodenal GIST demonstrate the feasibility of various surgical procedures: PD, pancreas-sparing duodenectomy, SD, or wedge local resection[8,9,11-17,22-25]. These papers can help us a little to determine which surgical procedure is optimal in terms of short- and long-term oncological results. The largest series of duodenal GISTs ($n = 156$) evaluated prognosis according to tumor grade[3]. In this pathological review, around 60% of patients underwent pancreas-preserving duodenectomy and 11% had PD, but due to the retrospective nature of this analysis, no correlation between type of operation and oncological results were reported. Very recently, Tien *et al*[26] have reported their experience, in which they compared nine PD with 16 limited operations (11 wedge resections, and five SDs) for duodenal GIST. They have shown that the type of operation is not correlated to operative risk or disease recurrence. They have concluded that limited procedures, like SD, should be attempted for duodenal GIST without involvement of the papilla of Vater. Others have reported similar results[10].

The choice of surgical procedure for duodenal GIST can be guided by the size and exact location of the tumor[6,11,17,25]. However, some principles of GIST surgical treatment have to be considered by a visceral surgeon when approaching duodenal GIST[1,4,6,10,17]. First, GISTs are mesenchymal tumors that behave as other sarcomas and not like adenocarcinomas[4,18]. GIST spreads specifically hematogenously and is rarely, if ever, associated with lymphatic invasion, as in other sarcomas[4,6,18]. Therefore, lymphadenectomy is not recommended[4,11,19]. This seems true for duodenal GIST as local lymph node invasion has never been described, even after PD[3,11-13,17]. Secondly,

GISTs are well encapsulated tumors that rarely show a tendency to local invasion even for high risk tumors[4,6,20]. They should be approached with the intention of performing complete *en bloc* removal (R0 resection) of the tumor and surrounding digestive tract tissue[1,4,6,7,10,19]. The size of surgical margins along the segment of digestive tract involved are not formally defined, however there is little submucosal spread in GIST and clear margins of 1 or 2 cm are recommended[4,6,19,20]. When extracapsular GIST mobilization is possible, there is no need for extensive surgical margins on adjacent organs and peri-tumoral resection with an intact capsule is sufficient[4,6,19,20].

Segmental or atypical duodenectomy for duodenal GIST is in accordance with these principles and could be beneficial for patients because it does not involve the excessive resection and morbidity associated with PD. After complete surgical resection, duodenal GIST prognosis seems not to be influenced by the pancreatic margins, according to the present study and the sparse literature on the subject. Prognosis is mainly dependent on malignant status, which is determined by size and mitotic rate (Fletcher scale). This has been clearly shown for duodenal GIST in the study by Miettinen *et al*[3] and was true in our small series. However, some authors have advocated the need for PD as pancreatic invasion cannot be ruled out on preoperative studies[17,27]. Although close contact between the GIST capsule and pancreas is usually the rule for large duodenal GISTs on CT (Figures 1 and 2), this is rarely correlated with pancreatic tumoral invasion, which allows treatment with pancreas-sparing duodenectomy[12,28,29]. As a result of these considerations, we think that segmental or atypical duodenectomy is the optimal procedure for duodenal GIST, as previously proposed by others[10-13,15,22-25,30]. The exact type of duodenectomy to perform might be influenced by GIST size and location, ranging from wedge resection with primary closure for small proximal duodenal GIST to SD with duodeno-jejunal anastomosis for large distal duodenal GIST[11,15,30-32]. One exception to this might be periampullary or ampullary GIST, which can present with jaundice, for which pancreas-preserving duodenectomy can be challenging compared to cephalic PD, when the ampulla needs to be resected to obtain clear surgical margins[8,27].

The present study has several limitations. First, it could be argued that a higher rate of malignant GIST is present in the PD group, because in part, PD was chosen for larger and more difficult-to-reach lesions. Furthermore, a longer median follow-up was available for the PD group. These two points could counter-balance the results. Finally, the sample was small, but duodenal GIST remains a rare tumor. Previous studies published in the literature have not reported large numbers of patients, and most of the time, only case reports[8-17,19,22-27,30].

In conclusion, pancreas-preserving segmental or atypical duodenectomy seems to be a reliable and curative option in duodenal GIST. Despite being limited in their extent, these methods of resection, when performed with negative margins, are compatible with long-term DFS, and should be preferred, whenever possible, to PD. This is related to the tumoral characteristics of GIST, which is generally well encapsulated, even when highly malignant and with extremely rare lymphatic spread. When clear surgical margins are achieved, prognosis depends on tumoral malignant potential and not on the extent of the surgical margins, especially the pancreatic margin, for duodenal GIST. However, PD remains a good alternative for tumors in the vicinity of the ampulla of Vater.

COMMENTS

Background

Duodenal gastrointestinal stromal tumors (GISTs) are rare primary duodenal tumors, and there are few guidelines to help the clinician in their surgical management. Surgery is still the only curative approach for GIST, but the optimal procedure remains to be established. Although, numerous authors have reported various surgical procedures, few have correlated their results with oncological outcomes.

Research frontiers

This study was designed to assess the optimal surgical procedure for duodenal GIST, and to compare segmental resection with more extensive pancreaticoduodenectomy (PD).

Innovations and breakthroughs

The authors reported good oncological outcomes with long-term disease-free survival (DFS) in the segmental duodenectomy (SD) group. Thus, whenever associated with clear surgical margins, SD is a reliable and curative option for most duodenal GISTs. However, PD remains a good alternative for tumors in the vicinity of the ampulla of Vater.

Applications

Segmental resection should be preferred, when possible, to more extensive procedures for duodenal GIST. However, for tumors located in the vicinity of the ampulla of Vater, PD remains a good option.

Peer review

The authors evaluated the results of SD (five cases) and PD (two cases) for duodenal GIST. The very low number of patients is certainly a weakness of this study; on the other hand, duodenal GIST is very rare. The authors obtained good DFS following limited resection (SD) with clear margins.

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BRIEF ARTICLE

Reduction of the closure time of postoperative enterocutaneous fistulas with fibrin sealant

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Abstract

AIM: To assess whether the use of fibrin sealant shortens the closure time of postoperative enterocutaneous fistulas (ECFs).

METHODS: The prospective case-control study included 70 patients with postoperative ECFs with an output of < 500 mL/d, a fistulous tract of > 2 cm and without any local complication. They were divided into study (n = 23) and control groups (n = 47). Esophageal, gastric and colocutaneous fistulas were monitored under endoscopic visualization, which also allowed fibrin glue application directly through the external hole. Outcome variables included closure time, time to resume oral feeding and morbidity related to nutritional support.

RESULTS: There were no differences in mean age, fistula output, and follow-up. Closure-time for all patients of the study group was 12.5 ± 14.2 d and 32.5 ± 17.9 d for the control group (P < 0.001), and morbidity related to nutritional support was 8.6% and 42.5%, respectively (P < 0.01). In patients with colonic fistulas, complete closure occurred 23.5 ± 19.5 d after the first application of fibrin glue, and spontaneous closure was observed after 36.2 ± 22.8 d in the control group (P = 0.36). Recurrences were observed in 2 patients because of residual disease. One patient of each group died during follow-up as a consequence of septic complications related to parenteral nutrition.

CONCLUSION: Closure time was significantly reduced with the use of fibrin sealant, and oral feeding was resumed faster. We suggest the use of fibrin sealant for the management of stable enterocutaneous fistulas.

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Key words: Enterocutaneous fistulas; Fibrin sealant; Spontaneous closure

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INTRODUCTION

The formation of enterocutaneous fistulas (ECFs) can be either spontaneous or as a consequence of intra-abdominal surgery. The incidence of spontaneous fistulas is around 15%-25%[1,2]. These can be secondary to Crohn's disease, malignancy, infectious diseases such as tuberculosis and deep mycosis, diverticulitis, vascular failure, radiation exposure and ischemia of the mesentery[3]. Postoperative fistulas account for 75%-85% out of all fistulas in the digestive tract and they arise from unintentional enterotomy, dehiscence of an anastomosis because of tension in the suture line, a foreign body located close to the anastomosis, inadequate suture techniques, distal obstructions, hematomas, abscess formation at the site of the anastomosis, or tumors[3-5]. Morbidity and mortality associated with postoperative fistulas are substantial as they are highly associated with nutritional deficits, septic complications and concomitant diseases that may appear during prolonged hospital stays[5-7]. The conventional treatment for postoperative fistulas includes intestinal rest, correction of electrolytic disturbances, parenteral nutrition, protection of the skin surrounding the fistula, and treatment and prophylaxis of any related local or systemic septic complications[5,6]. Spontaneous closure of ECFs occurs after 6-8 wk in 60%-70% of the cases after specific medical management[8]. Fistulas that do not close with conservative medical treatment require surgery[2,7].

Biological fibrin glues have long been used extensively in many surgical and speciality fields[9,10]. Fibrin glue contains high concentrations of human fibrinogen and thrombin and it has been widely applied clinically as a biological adhesive system for tissue adhesion or hemostasis[11-13]. Additionally, in comparison with other adhesives, this compound has several advantages in terms of biocompatibility, biodegradation and hemostasis[14-16]. Its use has also been extended for the management of untreatable fistulas that have not responded to conservative treatment[11,12]. The efficacy of fibrin varies depending on a number of features, such as the output volume of the fistula, the location of the fistula, and the presence of a fistulous tract long enough (greater than 2 cm) to allow fixation of the patch[17]. Following the placement of the fibrin patch, it is replaced by collagen after about 4 wk, leading to cessation of drainage and promotion of closure of the fistula[9,16] with avoidance of inflammatory processes, finally resulting in improved healing[18].

The aim of this study was to determine the outcomes and advantages of treating low-output-volume ECFs with the addition of fibrin glue and compare them with conservative management without the use of adjuvant application of fibrin glue into the fistulous tract.

MATERIALS AND METHODS

Over an 8-year period, 70 male and female adults with stable, non-complicated, low-output ECFs were included in this non-randomized prospective case-control study. During the same period, a total of 218 patients were evaluated for postoperative enterocutaneous fistulas. One hundred and forty-eight patients were not considered for the study because of high output volume (> 500 mL/24 h), abdominal infection, fistulous tract < 2 cm, entero-atmospheric fistulas and residual intestinal or peritoneal disease. Patients were divided into 2 groups, a study group (n = 23) in which patients received variable doses of fibrin glue through the external opening of the ECFs, and a control group (n = 47) of patients treated without fibrin glue. Both groups were assessed to compare endpoint variables. Patients belonging to the control group were selected according to the origin of the fistula, using 1 case per 2 controls. Both groups were treated with the same conservative management, known to promote spontaneous closure. Patients with any concomitant condition that impeded spontaneous closure were excluded from the study.

Evaluation of the external fistula and medical treatment

Patients with a fistula output volume of less than 500 mL/24 h for at least 3 consecutive days were chosen to participate in the study. We evaluated the characteristics of the fistulas with contrast fistulography, computed tomography (CT) scans and proximal or distal endoscopy to confirm the absence of any condition that might impede spontaneous closure of the fistula, such as complex tracts, associated abscesses, intra-abdominal sepsis, residual disease, foreign bodies or distal obstruction, and to determine the length of the fistulous tract. Those cases with purulent collections were treated with percutaneous drainage before the application of the fibrin glue. Fistulous tracts measuring less than 2 cm were not included in the study because it was not possible to produce an adequate adhesion area for the patch into the fistulous tract. Pure pancreatic fistulas were not included.

Conservative treatment consisted of fasting, suppression of gastric secretion with oral or intravenous omeprazole or ranitidine, subcutaneous octreotide and nutritional support, either parenteral or enteral according to the anatomic location of the fistula. All patients received skin protection with barriers to prevent burns. The most important endpoint variables were the changes in the output volume, the time required to reduce the output of the fistula to zero for 2 consecutive days, the time re-

Table 1 General characteristics of the patients with postoperative enterocutaneous fistulas and final results (mean ± SD)

Type of fistula	Sex (Male/female)	Age (yr)	Time of evolution (d)	Fistula output (mL/24 h)	Fistula closure time (d)	Restart oral intake (d)
Esophago-gastrocutaneous						
Study group (n = 3)	3/0	48.6 ± 12.2	23.3 ± 7	70 ± 42.7	8 ± 4	12 ± 3.6
Control group (n = 7)	7/0	49.1 ± 10.5	18.8 ± 4.4	88.1 ± 19	19.1 ± 6	24 ± 8.6
[1]P value		P = 0.85	P = 0.24	P = 0.15	P < 0.05	P < 0.05
Gastrocutaneous						
Study group (n = 5)	4/1	58.6 ± 9.4	93.8 ± 85.1	151.4 ± 146.1	7 ± 3.1	9.8 ± 2.4
Control group (n = 10)	6/4	47.4 ± 16.7	86.6 ± 69.8	131.4 ± 28.8	35.2 ± 18.7	39.7 ± 20.1
[1]P value		P = 0.19	P = 0.86	P = 0.67	P < 0.01	P < 0.01
Duodeno-jejunocutaneuos						
Study group (n = 5)	2/3	38.2 ± 10.4	25.6 ± 4.9	111.6 ± 75.9	7.6 ± 2.6	11.6 ± 4.3
Control group (n = 10)	6/4	44.1 ± 11.2	22.8 ± 4.2	123.6 ± 19.9	30 ± 17.1	34.9 ± 17.2
[1]P value		P = 0.34	P = 0.27	P = 0.63	P < 0.01	P < 0.01
Ileocutaneous						
Study group (n = 6)	2/4	53.1 ± 5	28.1 ± 9.6	132.8 ± 47.6	16.1 ± 21.6	18.3 ± 20.6
Control group (n =12)	6/6	55.4 ± 13.1	23 ± 4.7	143 ± 36	37.8 ± 17.2	42.5 ± 17.7
[1]P value		P = 0.69	P = 0.13	P = 0.61	P < 0.05	P < 0.05
Colocutaneous						
Study group (n = 4)	3/1	61.5 ± 8.3	40.2 ± 17	87.7 ± 40.8	23.5 ± 19.5	25 ± 17.8
Control group (n = 8)	5/3	60.1 ± 11.9	33.1 ± 13.2	106.1 ± 16.5	36.2 ± 22.8	40 ± 23.1
[1]P value		P = 0.84	P = 0.44	P = 0.28	P = 0.36	P = 0.25
All types of enterocutaneous fistulas						
Study group (n = 23)	14/9	51.9 ± 11.6	43.3 ± 46.3	116.2 ± 81.5	12.5 ± 14.2	15.3 ± 13.3
Control group (n = 70)	30/17	51.1 ± 13.7	37.5 ± 40.9	121.9 ± 31.5	32.5 ± 17.9	37.2 ± 18.4
[2]P value		P = 0.81	P = 0.59	P = 0.74	P = 0.000	P = 0.000

[1]P value from the Mann Whitney U test; [2]P value from the Student's t test.

quired to resume normal oral intake, and fistula reopening. In addition, we studied complications related to the use of nutritional support.

Fibrin glue application

To allow the adhesion of the fibrin glue patch, all fistulous tracts were debrided with an endoscopic brush to produce a smooth surface. The adhesive was composed of fibrinogen at a concentration of 80 mg/mL and 1000 IU/mL of thrombin (both of human extraction) combined with tranexamic acid and calcium chloride (Quixil®; Omrix Biopharmaceuticals Ltd., Tel-Hashomer, Israel). Prior to application, the adhesive was defrosted and components were placed separately in a double-syringe system with distal mixing device. The application of the glue through the external opening of the fistula was controlled endoscopically to assure total occlusion of the internal hole, in those patients with esophageal, gastric and colocutaneous fistulas. Patients with fistulas arising from the duodenum (after gastrectomy and gastrojejunostomy), jejunum and ileum, the control of the glue application was performed with fistulography to establish the distance between the internal and external holes, fistulous tract diameter and estimated volume of fibrin glue to administrate into the fistulous tract. We considered therapeutic failure when the fistula output persisted after the third application of the adhesive without a limit time.

Ethical considerations

This study was conducted according to the declaration of Helsinki of 1989 and Mexican Health Guidelines. The protocol was approved by the Ethical Committee of the Western National Medical Center at the Mexican Institute of Social Security. Full written informed consent was obtained from all patients before their inclusion to this study.

Statistical analysis

Results are described as percentages and central tendency and dispersion measures. Qualitative variables were analyzed using χ^2 or Fisher's exact test, and for quantitative variables the Mann Whitney U test or Student's t test was used according to the resulting distribution. All P values less than 0.05 were considered statistically significant.

RESULTS

Between January of 2000 and December of 2007, 70 patients were included. The study group consisted of 23 patients (14 male, 9 female) with a mean age of 51.95 ± 11.67 years, and a control group of 47 patients (30 male, 17 female) with a mean age of 51.17 ± 13.78 years. In both groups, all external fistulas were classified according to the anatomical origin (Table 1).

Esophago-gastrocutaneous fistulas

We included 10 patients with esophago-gastrocutaneous fistulas secondary to esophageal resection of distal carcinoma, caustic burns or high-grade dysplasia associated with Barrett's metaplasia. In all cases, the esophagus was replaced by gastric transposition through the posterior mediastinum. There were 3 cases in the study group and 7 in the control group. There was no difference in mean

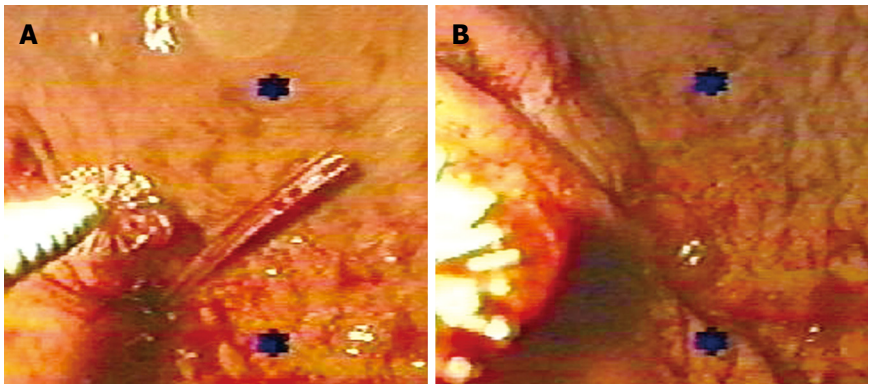


Figure 1 Endoscopic control of the application of fibrin glue in a patient with a gastrocutaneous fistula. A: The sealant was instilled externally; B: For better results, a complete occlusion of the internal hole of the fistulous tract is required.

age between groups (P = 0.85). The average output of the fistulas was 70 ± 42.7 mL and 88.1 ± 19 mL, respectively (P = 0.15). The fistulous tract measured 3.6 ± 0.36 cm and 3.65 ± 0.48 cm, respectively (P = 0.86). The mean quantity of fibrin glue applied into the external opening was 9 mL (range 7-11 mL). The time to obtain complete closure following the initial application was 8 ± 4 d in the study group. Spontaneous closure was observed after 19.1 ± 6 d of medical treatment in the control group (P < 0.05).

Gastrocutaneous fistulas

Five patients with gastrocutaneous fistulas were included in the study group and 10 in the control group. Gender distribution was similar in both groups and there were no differences in age (P = 0.19). Average fistula output was 151.4 ± 146.1 mL and 131.4 ± 28.8 mL (P = 0.67). The majority originated from endoscopic or surgical gastrostomies. One case in the control group had a fistula that originated from a partial dehiscence of a gastrojejunostomy. The fistulous tract measured 4.3 ± 0.77 cm and 4.1 ± 0.61 cm (P = 0.76) in study and control groups, respectively. The adhesive application was performed under endoscopic visual control to ensure complete occlusion of the internal hole (Figure 1). The mean quantity of fibrin glue was 18 mL (range 6-30 mL). In the study group, complete closure was observed 7.0 ± 3.1 d after the application. In the control group, spontaneous closure occurred after 35.2 ± 18.7 d (P < 0.01).

Duodenum and jejunocutaneous fistulas

Fifteen patients, 8 male and 7 female, were included in this group. One patient in the study group and 2 patients in the control group had pure duodenal fistulas after total or partial gastric resections. The remaining 12 cases had external jejunocutaneous fistulas, 4 in the study group and 8 in the control group, all of which resulted from partial dehiscence of jejunorrhaphy or anastomoses after resection. There were no differences in age between groups (P = 0.34). The mean fistula output was 111.6 ± 75.9 mL in the study group and 123.6 ± 19.9 mL in the control group (P = 0.63). The fistulous tract measured 6.5 ± 0.56 cm and 6.9 ± 0.68 cm (P = 0.26), respectively. The mean quantity of the fibrin glue applied into the external opening was 12.5 mL (range 10-15 mL). Complete closure was observed 7.6 ± 2.6 d after the application of the adhesive in the study group and at 30.0 ± 17.1 d in the control group (P < 0.01).

Ileocutaneous fistulas

Eighteen patients, 8 male and 10 female were included in this group, 6 in the study group and 12 in the control group. All fistulas were secondary to partial dehiscence of end-to-end anastomoses or enterorrhaphy. There was no age difference between the groups (P = 0.69). The average fistula output was 132.8 ± 47.6 mL *vs* 143.0 ± 36.6 mL in study and control groups, respectively (P = 0.61). The fistulous tract measured 10.4 ± 1.8 cm and 10.6 ± 1.9 cm, respectively (P = 0.85). The mean quantity of fibrin glue applied into the external opening was 14.5 mL (range, 10-19 mL). For the study group, complete closure was observed 16.1 ± 21.6 d after the first application and 37.8 ± 17.2 d in the control group (P < 0.05). In one of the patients the fibrin glue was applied endoscopically because the fistula originated from dehiscence of an ileocolic anastomosis 70 cm from the anal margin.

Colocutaneous fistulas

This group had 12 patients, 8 male and 4 female, 4 in the study group and 8 in the control group. There was no age difference between groups (P = 0.84). The average of the fistula output was 87.7 ± 40.8 mL in the study group and 106.1 ± 16.5 mL in the control group (P = 0.28). The fistulous tract measured 15.4 ± 2.1 cm and 14.1 ± 1.5 cm (P = 0.24). All fistulas were secondary to left colon resection, with manually constructed end-to-end anastomosis or primary closure after trauma. Diagnoses made before the resection were diverticular disease, sigmoid or proximal rectal cancer, and trauma with incidental perforation. The mean quantity of fibrin glue applied into the external opening was 14.5 mL (range 12-26 mL). In the study group, complete closure occurred 23.5 ± 19.5 d after the first application, and spontaneous closure was observed after 36.2 ± 22.8 d in the control group (P = 0.36).

Fistula recurrence, complications related to nutritional support and mortality

During an 18-mo follow-up period, reopening of fistulous tracts was observed in 2 patients with colocutaneous fistulas. Both patients belonged to the study group. One

patient suffered incidental perforation of the left colon during a necrosectomy with primary closure. After the reopening of the fistulous tract, endoscopic evaluation showed sigmoid stenosis, and a CT scan demonstrated a retroperitoneal inflammatory process probably related to a previous episode of acute pancreatitis, and after 8 mo he experienced spontaneous closure. The second case was a patient with residual diverticular disease of the left colon that was not identified during the initial evaluation before fibrin glue application. This patient underwent a segmental colon resection with subsequent resolution of the complication.

Morbidity related to nutritional support was significantly different between groups. In the study group, there was one minor and one major complication (8.6%). One patient required a change of jejunostomy tube because of obstruction, and the other patient, who had short bowel syndrome and was receiving long-term total parenteral nutrition, developed septic shock secondary to *Candida albicans* infection 11 mo after the resolution of the fistulous complication. This patient eventually died of multiple organ failure. In the control group, morbidity was observed in 20 patients (42.5%). This difference was statistically significant ($P < 0.01$). Minor complications such as tube obstruction, enteral nutrition-associated diarrhea, and central venous catheter obstruction were observed in 8 cases (17%). The remaining 12 patients developed major complications, such as fever related to a central venous catheter in 11 cases: 4 with negative cultures which were resolved with a new access; 7 patients with positive cultures, 6 with Gram-positive cocci (*Staphylococcus aureus* and *Streptococcus epidermidis*); and the remaining patient died of septic complications caused by a systemic *C. albicans* infection. There were 2 additional deaths during the follow-up from non-related causes, including one patient from each group (acute myocardial infarction and diffuse abdominal carcinomatosis). There were no complications related to the application of the fibrin glue. Finally, when we considered all patients stratified only by the interventional maneuver, we observed highly significant results. The time to achieve total fistula closure was 12.5 ± 14.2 d in the study group (fibrin glue) and 32.5 ± 17.9 d in the control group ($P < 0.001$).

DISCUSSION

Fibrin sealant, also referred to as fibrin glue or fibrin tissue adhesive, is a surgical hemostatic agent derived from plasma coagulation proteins[19]. Fibrin sealants are widely used for many surgical procedures in all fields of surgery[20]. They can be used for hemostasis, wound closure, and tissue sealing, and in contrast to synthetic adhesives, they have the advantage of being biocompatible and biodegradable, and they are not associated with inflammatory processes, foreign body reactions, tissue necrosis, or extensive fibrosis. With normal wound healing, fibrin absorption occurs within days to weeks of application, depending on the type of surgery, the proteolytic activity at the treated site, and the amount of sealant used[21].

Commercial concentrates containing human fibrinogen, factor XIII and bovine thrombin became available in Europe in the late 1970s and have been extensively used since then for hemostasis and other indications[22]. All these products contained antifibrinolytic agents. However, when first introduced, fibrin sealants were excluded from use in the United States based on the risk of transmission of infection[21,22]. The risk of viral transmission was reduced through careful donor selection followed by heat treatment of the human fibrinogen component. Virally inactivated human thrombin has replaced bovine thrombin in most European products[23]. A number of researchers have described the postoperative development of antibodies to bovine thrombin in some patients[24].

The first new generation fibrin sealants used in the United States, with removed or inactivated viruses, was Tisseel (Baxter Healthcare), which was approved by the US Food and Drug Administration (FDA) in 1998[25]. Tisseel was used in the majority of the studies performed to assess the use of this adhesive. In the present study, we used a fibrin sealant known as a second-generation sealant, which received FDA approval in March 2003 and is known as Crosseal (American Red Cross)[26]. Both Tisseel and Quixil are composed of human fibrinogen and human thrombin, which are combined at the time of use. The respective formula differs from each other because Tisseel contains bovine aprotinin (BA), while Quixil contains tranexamic acid, both known as antifibrinolytic agents[26].

Adverse events associated with the use of fibrin sealant over the least 25 years have been inflammatory processes, allergic reactions and viral infections secondary to the bovine component[27].

Hino *et al*[28] recently reported 3 cases of iatrogenic parvovirus B19 infection after the use of a fibrin sealant. This viral transmission was attributed to the use of dry-heat viral inactivation, which is not effective against non-enveloped viruses. Thus far, there are no reports of human immunodeficiency virus seroconversion or hepatitis B or C infection after the use of fibrin sealant[29]. However, the risk of virus transmission by fibrin sealants is still a subject of debate[19]. Three cases of anaphylaxis have been reported in Japanese patients, one of which was linked to the bovine aprotinin component of the sealant[30].

Fibrin adhesives have been used for several decades to close or obliterate fistulous tracts[31] and their effectiveness varies depending on the output volume of the fistula, its location, and the presence of a sufficiently long fistulous tract (greater than 2 cm) to allow fixation of the patch[32]. Postoperative fistulas of the digestive tract are relatively frequent in surgical practice. Serious technical difficulties such as variability in the location of the fistulas, quantification of drainage, time of evolution of the fistula, patient status and underlying pathology, have hindered scientific research comparing various therapeutic options and treatments[32]. This is one of the reasons why there are just a few randomized controlled clinical trials with the use of fibrin sealants[19].

In 1996, Hwang *et al*[32], published a small randomized clinical trial in patients with very low-output enterocutaneous fistulas (less than 20 mL). They used fibrin glue extracted from a blood bank and activated with bovine thrombin. The anatomic origin of the fistulas was varied and included upper and lower digestive tract fistulas. In 6 patients treated with external application of the adhesive, the time needed to close the fistulous tract was reduced significantly (2 ± 0.4 d), as was the hospital stay. Control patients (n = 7) received conventional treatment with total parenteral nutrition. In this group, spontaneous closure was observed after 13 ± 2 d ($P < 0.01$). The authors concluded that the use of fibrin glue is safe and effective for patients with stable and low-output enterocutaneous fistulas.

Waag *et al*[33] performed the first endoscopic application of an adhesive in 1979, for the treatment of a tracheo-esophageal fistula. Subsequently, with the introduction of different types of biological tissue adhesives and the development of dual and triple lumen catheters in 1984, fibrin glues have been used in the endoscopic treatment of fistulas of the digestive tract[34]. However, only proximal and distal digestive tract fistulas are accessible for endoscopic evaluation and management. In 1990, Nakagawa *et al*[35] reported the use of a small-diameter endoscope for the evaluation and treatment of fistulous tracts with fibrin glue. Fifteen patients were included in this study, and they were submitted to endoscopic examination, the clinical significance of which was assessed, and it was concluded that fistuloscopy is a safe and easy technique that results in less stress for the patients and is considered to be effective for the examination and treatment of fistulas[35].

Lange *et al*[36] investigated whether the endoscopic procedure could be used as an adjuvant technique for the sealing of gastrointestinal fistulas in 17 enterocutaneous fistulas. The success rate was 64.5%, and they described complementary treatment for abscesses associated with the fistulous tract. Some patients developed complications because of the high pressure of the fibrin glue application, and one patient died of pulmonary air embolism[36].

As reported by Shand *et al*[37] in 1997, some adjuvants have been purported to favor the early closure of fistulous tracts. They described a case of a patient with a non-healing low-output gastrocutaneous fistula closed by endoscopic means with fibrin glue and surgical packs. The fistula reopened 3 d after the procedure, and closure of the fistula was reattempted by direct endoscopic application of surgical packs and adhesive. Complete closure was obtained 11 d after direct endoscopic application of the fibrin glue[37].

Recently, Lomis *et al*[38] performed a study in which they evaluated 7 patients with persistent fistula, treating them with collagen plugs. Under fluoroscopic guidance and using direct-catheter techniques, collagen plugs were applied into the fistulas. The success rate was 85.7%; 6 of the 7 patients had resolution of the fistula with no evidence of fistula recurrence 30-180 d after the closure.

In 1990, Eleftheriadis *et al*[39] reported 7 cases of proximal digestive fistulas with high-volume output (700 mL in 24 h minimum) that were closed with fibrin gel by endoscopic means, with a 100% success rate. In 2002, the same authors reported a study in 14 patients; 7 with low-volume-output fistulas (discharging 20-50 mL/d) and 7 with high-volume-output fistulas (200-1000 mL/d), using fistuloscopy to evaluate the fistulous tracts and to measure the length of the fistula, with removal of necrotic material and non-absorbable sutures. Fibrin sealant was applied under direct observation. The fistulas healed within 2-17 d (mean 9.2 ± 5.1 d) in all patients except one with peritoneal carcinomatosis. The authors concluded that fistuloscopy could be used as both a diagnostic and a therapeutic tool in low- and high-output postoperative fistulas resistant to conservative treatment[40]. More recently, in 2004, the same author reported the endoscopic application of fibrin sealant for the treatment of gastrocutaneous fistula after bariatric surgery in three morbidly obese patients[41].

Kurokawa *et al*[42], reported an 85% success rate after the selective occlusion of complex digestive fistulas with fibrin glue application under fistuloscopy in patients in whom fistula closure had not occurred after 3 wk of conservative postoperative treatment.

Ramón Rábago *et al*[43], recently reported the largest series of postoperative digestive fistulas treated with transendoscopic application of fibrin glue. They included 30 patients with proximal, distal and internal fistulas of low- and high-output. The medium time of conservative treatment was 95 ± 199 d for all types of digestive fistulas. The success rate was 80% and 25% respectively and 55% for internal fistulas. They recommended that conservative treatment should not be prolonged beyond 2-4 wk, since most postoperative fistulas required 14 d to stabilize, and that endoscopic treatment should be performed at that stage. After follow-up periods ranging from 6 mo to more than 6 years, only one of the sealed fistulas reopened requiring a new endoscopic resealing. Rabago and his colleagues concluded that endoscopic treatment achieves a very high success rate, without complications and at a lower cost[43].

The first Mexican experience with fibrin sealants was published in 1997 by Justo-Janeiro *et al*[44]. They evaluated the application of concentrated human fibrinogen activated with bovine thrombin, fundamentally for hemostatic purposes, protection of high-risk anastomosis, seroma prevention and closure of enterocutaneous fistulas (4 patients). The success rate in the early closure of fistulous tracts was limited (50%), and failures were a consequence of distal obstruction to the fistula origin and intense inflammatory reactions[44].

Since 1998 commercially-prepared fibrin glue became available in our country and in 1999 our group designed protocols to evaluate the efficacy of the fibrin sealant in different surgical indications, including general surgery. In this study, we used human fibrin glue exclusively in low-output digestive fistulas, and we made comparisons with patients who received the same medical treatment but without the use of the adhesive. Our results are relevant

as the closure times achieved were significantly reduced, and the time needed to resume oral intake was also decreased. Application through the external opening is very useful when the characteristics of the fistulous tract are well known. Recently, Murakami *et al*[45], demonstrated adequate closure of complex postoperative digestive fistulas identifying the characteristics of the fistulous tract with an injection of contrast medium through the drainage tube. Eighteen patients were included in the study, with an 88% success rate (16 patients) with the applications of 1 to 9 treatment sessions of diluted solution of thrombin and fibrinogen 80 mg/mL. They studied the effect of thrombin dilution in saline solution and found the optimal concentration at 8 mg/mL of thrombin with a coagulation time of 45 ± 5 s. The delayed coagulation time permitted injection of the glue through all the complex fistulous tracts reaching a high success rate despite 60% of patients having abscesses associated with the fistulous tracts. The treatment was offered between 31 and 168 (66 ± 40.4) d after the surgical procedures.

In the series presented here, endoscopic control of the applications was performed in 13 cases with proximal or distal fistulas. The persistence of inflammation and inadvertent diverticular disease induced the recurrence of 2 colonic fistulas which eventually resolved with medical and surgical treatment. We attribute the success rate, even without performing a fistuloscopy or transendoscopic application, to the meticulous instillation of the fibrin glue, the low-volume output of the enterocutaneous fistulas, and the absence of complex fistulous tracts or any other condition that impeded spontaneous closure of the fistulas, such as associated abscesses, intra-abdominal sepsis, residual disease and foreign bodies or distal obstruction. Morbidity related to nutritional support was higher in the control group due to the prolonged time to feeding, and mortality was secondary to catheter-related sepsis in chronic parenteral nutrition. Since accelerated closure was not the purpose of this study, we proposed to treat patients as soon as the fistulas became stable and low-output without any associated obstruction, infection or residual disease, as other authors suggested in treatment of this complication[32,43,45].

In summary, we recommend the use of fibrin glue in those patients who do not experience spontaneous closure, to reduce the time needed to complete the resolution of the fistula and to minimize the rate of complications related to the secretions of the fistulas. The sealant should be applied as soon as the fistula becomes stable with the lowest output to assure closure after one or more applications of the glue.

Endoscopic control of the fibrin glue application should be performed whenever possible to ensure complete occlusion of the internal hole. The technique employed in this study has the advantage of allowing the removal of necrotic tissue and non-absorbable sutures from the fistulous tracts. Radiological visualization of the fistulous tracts, as studied here, requires less technological support, and we successfully treated digestive fistulas originating from the small bowel, including the duodenum, with external application of the adhesive. Patients did not present any adverse reactions, and recurrences were attributed to residual or untreated disease, particularly in those patients with colonic fistulas.

COMMENTS

Background

The formation of enterocutaneous fistulas can be either spontaneous or as a consequence of intra-abdominal surgery. The incidence of spontaneous fistulas is around 15%-25%, and postoperative fistulas account for 75%-85% of all fistulas of the digestive tract. Morbidity and mortality associated with postoperative fistulas are substantial as they are highly associated with nutritional deficits, septic complications and concomitant diseases that may appear during prolonged hospital stays. The conventional treatment for postoperative fistulas includes intestinal rest, correction of electrolytic disturbances, parenteral nutrition, and protection of the skin surrounding the fistula, and treatment and prophylaxis of any related local or systemic septic complications. Spontaneous closure occurs after 6-8 wk in 60%-70% of cases after specific medical management.

Research frontiers

Biological fibrin glues have long been used extensively in many surgical and specialty fields. Its use has also been extended for the management of untreatable fistulas that have not responded to conservative therapy. The efficacy of fibrin varies depending on the features of the fistula, such as output volume, location, and the presence of a fistulous tract long enough (> 2 cm) to allow fixation of the patch. Following the placement of the fibrin patch, it is replaced by collagen, leading to cessation of drainage and closure of the fistula with avoidance of inflammatory processes, finally resulting in improved healing.

Innovations and breakthroughs

This study determined that the application of fibrin glue through the external opening of stable enterocutaneous fistula reduced the closure time and the morbidity associated with nutritional support.

Applications

Instillation of variable quantities of fibrin glue (6 to 30 mL) according to the fistulous tract in one to 3 treatment sessions. The application of the glue was controlled endoscopically to assure total occlusion of the internal hole in proximal and distal fistulas.

Peer review

This is a good paper which targets a difficult and important topic.

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BRIEF ARTICLE

Post-gastrectomy spleen enlargement and esophageal varices: Distal *vs* total gastrectomy

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Abstract

AIM: To study the relationship between platelet count-to-spleen diameter ratio and post-gastrectomy esophageal varices (EVs) development in patients without liver cirrhosis or hepatitis.

METHODS: We retrospectively studied 92 patients who underwent gastrectomy. They were divided into 2 groups on the basis of the surgical treatment: the distal gastrectomy (DG) group and total gastrectomy (TG) group. The incidence of EVs was determined and postoperative platelet counts, spleen diameters, and platelet count-to-spleen diameter ratios were compared between the 2 groups.

RESULTS: EVs were not detected during the first 6 mo after surgery in either group; however, at 12 mo after surgery, EVs were detected in 2 patients (3%) in the DG group and in 1 patient (3.6%) in the TG group; their mean platelet count-to-spleen diameter ratio was 2628 ± 409, and 2604 ± 360, respectively.

CONCLUSION: Endoscopy should be performed to detect EVs when the platelet count-to-spleen diameter ratio is < 2600.

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Key words: Spleen enlargement; Esophageal varices; Platelet count; Distal gastrectomy; Total gastrectomy

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INTRODUCTION

Esophageal varices (EVs) are one of the major life-threatening complications of liver cirrhosis[1], and its prevalence is approximately 40% at the time of diagnosis, and 60% in those with decompensated disease[2,3]. When EVs rupture, the mortality rate ranges from 17% to 57%[4-7]. Therefore, screening of all patients diagnosed with liver cirrhosis for the presence of EVs is recommended[8,9]. Giannini *et al*[10] performed a study to identify parameters that could aid noninvasive prediction of EVs, and reported that the platelet count-to-spleen diameter ratio was the best noninvasive predictor of EVs. However, spleen enlargement is frequently observed during follow-up of noncirrhotic patients who have

undergone gastrectomy, and, in addition, some of these patients developed EVs. Thus, we studied the relationship between the platelet count-to-spleen diameter ratio and the development of EVs, and compared distal and total gastrectomy (TG) with regard to these variables in patients without liver cirrhosis or hepatitis.

MATERIALS AND METHODS

Patients

We retrospectively studied 92 patients (66 men and 26 women; age range, 30-80 years; mean age, 68 ± 10 years) who underwent gastrectomy between May 2002 and April 2006 at the Department of Surgery, Social Insurance Yokohama Central Hospital, Yokohama, Japan. The following patients were excluded from our study: those who had undergone abdominal operation in the past, those with hepatitis, and those in whom disease recurrence was observed. Preoperative upper gastrointestinal endoscopy was performed in all the patients, further, EVs were not confirmed in any of the patients. These patients were divided into the following 2 groups on the basis of the surgical treatment they received: patients who underwent distal gastrectomy (DG), and patients who underwent TG. The maximum bipolar diameter of the spleen was measured by ultrasound scanning. The platelet count, spleen diameter, and platelet count-to-spleen diameter ratio were measured after surgery, and their relationship with the occurrence of EVs was studied.

Endoscopic classification of esophageal varices

On the basis of the endoscopic findings, the EVs were classified into 3 grades: grade 1, varices could be compressed with the endoscope; grade 2, varices could not be compressed with the endoscope; and grade 3, varices were confluent around the esophagus[11].

Statistical analysis

Univariate analysis was performed using the Student *t* test for continuous variables and Fisher's exact test and the χ^2 test for categorical variables. A *P*-value of less than 0.05 was considered to be significant.

RESULTS

Table 1 shows the patient characteristics and preoperative variables. No differences were observed between the 2 groups with respect to mean age, sex ratio, and preoperative clinical data. The gastric cancers were staged according to the Japanese Classification of Gastric Carcinoma[12], and their stages were found to be similar.

Table 2 shows the variables 3 mo after the operation. The mean platelet count, mean spleen diameter, and mean platelet count-to-spleen diameter ratio were 25.8 × 10^4 ± 4.2 × 10^4/μL, 93.9 ± 7.6 mm, and 2769 ± 453, respectively, in the DG group, and 25.1 × 10^4 ± 3.9 × 10^4/μL, 96.8 ± 9.5 mm, and 2593 ± 328, respectively, in the TG group. No differences were observed between the 2 groups, and no EVs developed in either group.

Table 1 Characteristics of patients *n* (%), (mean ± SD)

	DG group (*n* = 64)	TG group (*n* = 28)	*P*-value
Age (yr)	68.3 ± 10.1	69.3 ± 6.7	0.6522
Sex ratio (male:female)	46:18	20:8	0.9651
Stage			
ⅠA	15 (23.4)	5 (17.9)	0.5504
ⅠB	13 (20.3)	7 (25)	0.6160
Ⅱ	16 (25.0)	7 (25)	1.00
ⅢA	17 (26.6)	7 (25)	0.8752
ⅢB	3 (4.7)	2 (7.1)	0.6382
Initial disease			
Hypertension	10 (15.6)	3 (10.7)	0.7474
Diabetes mellitus	9 (14.1)	3 (10.7)	0.7502
Atrial fibrillation	2 (3.1)	1 (3.6)	1.00
Apoplexy	2 (3.1)	0 (0)	0.5184
Parkinson disease	1 (1.6)	0 (0)	0.3043
Old pulmonary tuberculosis	4 (6.3)	1 (3.6)	1.00
Preoperative platelet count (× 10^4/μL)	24.8 ± 4.1	24.5 ± 3.4	0.7386
Preoperative spleen diameter (mm)	90.8 ± 8.5	91.9 ± 10.4	0.5908
Preoperative Platelet count/spleen diameter ratio	2744 ± 473	2674 ± 369	0.4921

DG: Distal gastrectomy; TG: Total gastrectomy.

Table 2 Clinical data 3 mo postoperatively *n* (%), (mean ± SD)

	DG group (*n* = 64)	TG group (*n* = 28)	*P*-value
Platelet count (× 10^4/μL)	25.8 ± 4.2	25.1 ± 3.9	0.4580
Spleen diameter (mm)	93.9 ± 7.6	96.8 ± 9.5	0.1251
Platelet count/spleen diameter ratio	2769 ± 453	2593 ± 328	0.0674
Esophageal varices			
Grade 1	0 (0)	0 (0)	1.00
Grade 2	0 (0)	0 (0)	1.00
Grade 3	0 (0)	0 (0)	1.00
Total occurrence rate	0 (0)	0 (0)	1.00

Grade 1: The varices could be depressed by the endoscope; Grade 2: The varices could not be depressed by the endoscope; Grade 3: The varices were confluent around the esophagus.

Table 3 shows the variables 6 mo after the operation. The mean platelet count, mean spleen diameter, and mean platelet count-to-spleen diameter ratio were 26.7 × 10^4 ± 4.3 × 10^4/μL, 95.6 ± 7.6 mm, and 2853 ± 458, respectively, in the DG group, and 26.5 × 10^4 ± 3.9 × 10^4/μL, 100.6 ± 9.4 mm, and 2632 ± 373, respectively, in the TG group. There was no significant difference between the 2 groups with respect to the platelet count; however, the mean spleen diameter in the TG group was significantly greater than that in the DG group ($P < 0.0078$), and the mean platelet count-to-spleen diameter ratio in the TG group was significantly lower than that in the DG group ($P < 0.0274$). No EVs developed in the patients in either group.

Table 4 shows the variables 12 mo after the operation. The mean platelet count, mean spleen diameter, and mean platelet count-to-spleen diameter ratio were 26.8 × 10^4 ± 3.9 × 10^4/μL, 102.4 ± 8.7 mm, and 2628 ± 409, respectively, in the DG group, and 27.5 × 10^4 ± 3.7 × 10^4/μL, 105.9 ± 9.0 mm, and 2604 ± 360, respective-

Table 3 Clinical data 6 mo postoperatively *n* (%), (mean ± SD)

	DG group (*n* = 64)	TG group (*n* = 28)	*P*-value
Platelet count (× 10^4/μL)	26.7 ± 4.3	26.5 ± 3.9	0.8016
Spleen diameter (mm)	95.6 ± 7.6	100.6 ± 9.4	0.0078
Platelet count/spleen diameter ratio	2853 ± 458	2632 ± 373	0.0274
Esophageal varices			
Grade 1	0 (0)	0 (0)	1.00
Grade 2	0 (0)	0 (0)	1.00
Grade 3	0 (0)	0 (0)	1.00
Total occurrence rate	0 (0)	0 (0)	1.00

Table 4 Clinical data 12 mo postoperatively *n* (%), (mean ± SD)

	DG group (*n* = 64)	TG group (*n* = 28)	*P*-value
Platelet count (× 10^4/μL)	26.8 ± 3.9	27.5 ± 3.7	0.4358
Spleen diameter (mm)	102.4 ± 8.7	105.9 ± 9.0	0.0843
Platelet count/spleen diameter ratio	2628 ± 409	2604 ± 360	0.7887
Esophageal varices			
Grade 1	2 (3)	1 (3.6)	1.00
Grade 2	0 (0)	0 (0)	1.00
Grade 3	0 (0)	0 (0)	1.00
Total occurrence rate	2 (3)	1 (3.6)	1.00

Table 5 Clinical data 24 mo postoperatively *n* (%), (mean ± SD)

	DG group (*n* = 64)	TG group (*n* = 28)	*P*-value
Platelet count (× 10^4/μL)	26.4 ± 3.7	25.8 ± 4.0	0.4533
Spleen diameter (mm)	104.4 ± 9.8	109.9 ± 10.1	0.0164
Platelet count/spleen diameter ratio	2546 ± 380	2357 ± 365	0.00287
Esophageal varices			
Grade 1	2 (3)	2 (7.1)	0.5825
Grade 2	0 (0)	1 (3.6)	0.3043
Grade 3	0 (0)	0 (0)	1.00
Total occurrence rate	2 (3)	3 (10.7)	0.1629

Table 6 Clinical data 36 mo postoperatively *n* (%), (mean ± SD)

	DG group (*n* = 64)	TG group (*n* = 28)	*P*-value
Platelet count (× 10^4/μL)	26.3 ± 3.8	25.5 ± 4.3	0.3904
Spleen diameter (mm)	105.6 ± 9.8	110.6 ± 9.6	0.0147
Platelet count/spleen diameter ratio	2515 ± 386	2317 ± 381	0.0256
Esophageal varices			
Grade 1	3 (4.7)	3 (10.7)	0.3638
Grade 2	1 (1.6)	1 (3.6)	0.5184
Grade 3	0 (0)	0 (0)	1.00
Total occurrence rate	4 (6.3)	4 (14.3)	0.2082

ly, in the TG group. No differences were observed between the 2 groups. However, 2 patients (3%) in the DG group and 1 patient (3.6%) in the TG group had grade 1 EVs. There was no significant difference between the 2 groups with respect to the occurrence of EVs.

Table 5 shows the variables 24 mo after the operation. The mean platelet count, mean spleen diameter, and mean platelet count-to-spleen diameter ratio were 26.4 × 10^4 ± 3.7 × 10^4/μL, 104.4 ± 9.8 mm, and 2546 ± 380, respectively, in the DG group, and the corresponding values were 25.8 × 10^4 ± 4.0 × 10^4/μL, 109.9 ± 10.1 mm, and 2357 ± 365, respectively, in the TG group. No differences were observed between the 2 groups with respect to the platelet count; however, the mean spleen diameter in the TG group was significantly greater than that in the DG group ($P < 0.0164$), and the mean platelet count-to-spleen diameter ratio in the TG group was significantly lower than that in the DG group ($P < 0.00287$). Two patients (3%) in the DG group and 2 patients (7.1%) in the TG group had grade-1 EVs, and 1 patient (3.6%) in the TG group had grade 2 EVs; however, there was no significant difference between the 2 groups with respect to the occurrence of EVs.

Table 6 shows the variables 36 mo after the operation. The mean platelet count, mean spleen diameter, and mean platelet count-to-spleen diameter ratio were 26.3 × 10^4 ± 3.8 × 10^4/μL, 105.6 ± 9.8 mm, and 2515 ± 386, respectively, in the DG group, and 25.5 × 10^4 ± 4.3 × 10^4/μL, 110 ± 9.6 mm, and 2317 ± 381, respectively, in the TG group. No differences were observed between the 2 groups with respect to the platelet count; however, the mean spleen diameter in the TG group was significantly greater than that in the DG group ($P < 0.0147$), and the mean platelet count-to-spleen diameter ratio in the TG group was significantly lower than that in the DG group ($P < 0.0256$). However, 3 patients (4.7%) in the DG group and 3 patients (10.7%) in the TG group had grade 1 EVs, and 1 patient (1.6%) in the DG group and 1 patient (3.6%) in the TG group had grade 2 EVs. There was no significant difference between the 2 groups with respect to the occurrence of EVs.

DISCUSSION

Bleeding from ruptured EVs is the leading cause of death among patients with liver cirrhosis[1], and the mortality rate from this complication varies between 17% and 57%[4-7]. Thus, cirrhotic patients should be screened for the presence of EVs. Some authors suggested that repeated endoscopy at intervals of 2-3 years should be performed in patients without varices, and that it should be performed at intervals of 2 years in patients with small varices in order to evaluate the development or progression of EVs[13,14]. However, in order to develop less invasive and cost-effective screening procedures for EVs, several studies have attempted to validate parameters that could be used for noninvasive screening[15-21], and have found that platelet count and spleen diameter showed a good correlation with the presence of EVs. In addition, Giannini *et al*[10] and Baig *et al*[22] reported that

the platelet count-to-spleen diameter ratio has the highest accuracy for noninvasive prediction of EVs in cirrhotic patients. However, spleen enlargement is frequently observed after patients have undergone gastrectomy. Thus, we studied the relationship between the platelet count-to-spleen diameter ratio and the presence of EVs after gastrectomy, and compared distal and TG with regard to these parameters in patients without liver cirrhosis or hepatitis. In addition, in order to avoid surgical influence, patients who had undergone abdominal operation in the past were excluded from this study. All our patients were healthy and did not exhibit any recurrence during their follow-up. In patients who did not have liver cirrhosis or hepatitis, the occurrence rate of EVs was thought to be low; however, splenic arterial flow was thought to increase after but not before the surgery. In general, blood flow in the celiac artery (CA) is diverted into 3 arteries: the common hepatic artery (CHA), left gastric artery (LGA), and splenic artery (SA). During gastrectomy (distal and total), the LGA and left gastric vein are ligated and cut, and blood flow from the CA is diverted into 2 arteries - the CHA and SA. Thus, blood flow in the CHA and SA increases after surgery. Moreover, during lymph node dissection around the celiac axis, collateral veins from the splenic vein are ligated and cut; thus, in our patients, blood outflow from the spleen was thought to decrease after the surgery as compared to that before the surgery. Also, in the TG group, short gastric veins were ligated and cut, and blood outflow from the spleen was thought to be lower than that in the GD group patients. This may be one of the main reasons why spleen enlargement was greater in the TG group than in the DG group.

Giannini *et al*[10] reported that the prevalence-adjusted positive and negative predictive values for a platelet count-to-spleen diameter ratio of 909 were 96% and 100%, respectively, and Baig *et al*[22] reported that the prevalence-adjusted positive and negative predictive values for a platelet count-to-spleen diameter ratio of 1014 were 9.4% and 95.1%, respectively. In our study, grade 1 EVs were detected in 2 patients (3%) in the DG group, and in 1 patient (3.6%) in the TG group at 12 mo after surgery, and the mean platelet count-to-spleen diameter ratio of the patients was found to be 2628 ± 409 in the DG group, and 2604 ± 360 in the TG group. At 24 mo after surgery, grade 2 EVs were detected in 1 patient (3.6%) in the TG group, and the mean platelet count-to-spleen diameter ratio of these patients was found to be 2357 ± 365. In the DG group, grade 2 EVs were detected 36 mo after the surgery, and the mean platelet count-to-spleen diameter ratio of the patients was found to be 2515 ± 386. In our study, EVs developed in 4 patients each in the DG group (6.3%) and the TG group (14.3%) after the surgery; however, no patient exhibited grade 3 EVs. In addition, none of our patients had liver cirrhosis or hepatitis; this contributed to the higher platelet count-to-spleen diameter ratio than that reported by Giannini *et al*[10] and Baig *et al*[22]. To the best of our knowledge, studies on the relationship between the platelet count-to-spleen diameter ratio and the presence of EVs have not been conducted in patients who underwent gastrectomy. Thus, on the basis of the results of our study, we suggest that the occurrence rate of EVs may increase after 6 mo post DG and when the platelet count-to-spleen diameter ratio is less than 2600. Thus, in the case of patients who have undergone gastrectomy for gastric cancer, the development of EVs after surgery is a strong possibility.

In conclusion, spleen enlargement after gastrectomy is greater in the case of TG than DG, and the platelet count-to-spleen diameter ratio is a useful parameter for the noninvasive prediction of EVs after gastrectomy. In addition, we think that the occurrence rate of EVs increases after 6 mo post gastrectomy, and therefore, when the platelet count-to-spleen diameter ratio is less than 2600, endoscopy should be performed to determine the presence of EVs.

COMMENTS

Background

Esophageal varices (EVs) are one of the major life-threatening complications of liver cirrhosis, and its prevalence rate is approximately 40% at the time of diagnosis, and 60% in those with decompensated disease. Therefore, screening of all patients diagnosed with liver cirrhosis for the presence of EVs is recommended.

Research frontiers

Spleen enlargement is frequently observed during follow-up of noncirrhotic patients who have undergone gastrectomy. The authors studied the relationship between the platelet count-to-spleen diameter ratio and the development of EVs, and compared distal and total gastrectomy (TG) with regard to these variables in patients without liver cirrhosis or hepatitis.

Innovations and breakthroughs

The authors retrospectively studied 92 patients who underwent gastrectomy. They were divided into 2 groups on the basis of the surgical treatment: the distal gastrectomy (DG) group and TG group. The incidence of esophageal varices was determined and postoperative platelet counts, spleen diameters, and platelet count-to-spleen diameter ratios were compared between the 2 groups.

Applications

Spleen enlargement after gastrectomy is greater in the case of TG than DG, and the platelet count-to-spleen diameter ratio is a useful parameter for the noninvasive prediction of EVs after gastrectomy.

Peer review

It is an interesting manuscript, though the background did not identify the real reason why the authors were embarking on their endeavor. The results were well presented and the discussion would be improved. It is a nice experiment.

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BRIEF ARTICLE

Recurrence after endoscopic piecemeal mucosal resection for large sessile colorectal polyps

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Abstract

AIM: To evaluate the safety and outcomes of endoscopic piecemeal mucosal resection (EPMR) for large sessile colorectal polyps.

METHODS: The patients enrolled in this study were 47 patients with 50 large sessile polyps (diameter, 2 cm or greater) who underwent EPMR using a submucosal saline injection technique between December 2002 and October 2005. All medical records, including characteristics of the patients and polyps, complications, and recurrences, were retrospectively reviewed. The first follow-up endoscopic examination was performed at 3-6 mo after initial endoscopic resection, and the second at 12 mo post-EPMR. Subsequent surveillance colonoscopic examinations were individualized, taking risk factors into account.

RESULTS: The patients were 23 men and 24 women, with a mean age of 60 years. Mean polyp size was 30.1 mm. Of 50 polyps identified, 34 (68%) were benign and 16 (32%) were malignant. There were 6 (12%) cases with EPMR-related bleeding: 5 intra-procedural and 1 early post-procedural bleeding. All bleeding episodes were managed by endoscopic clipping or argon beam coagulation. There were no perforations. Recurrence was identified in 5 cases (12.2%): 4 local recurrences detected at 3 mo post-EPMR and 1 local recurrence detected at 14 mo post-EPMR. The recurrence rate after EPMR was 3.1% for benign polyps and 33.3% for malignant polyps ($P < 0.05$). Median follow-up time was 37 mo.

CONCLUSION: EPMR is safe, but should be applied carefully in malignant polyps. Close follow-up endoscopic examinations are necessary for early detection of recurrence.

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Key words: Colonoscopy; Colorectal neoplasm; Endoscopic piecemeal mucosal resection; Large sessile polyps

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INTRODUCTION

Endoscopic resection of large sessile colorectal polyps

remains challenging because of its technical difficulty and high complication rate[1]; even so, the endoscopic removal of colonic adenomatous polyps is a commonly used technique that reduces the incidence of colorectal cancer. Some investigators have reported that endoscopic piecemeal mucosal resection (EPMR) is a safe and effective procedure for large sessile colorectal polyps[2-4]; however, this approach remains controversial because of the high possibility of coexisting malignancy and a high recurrence rate associated with large sessile polyps. Endoscopic submucosal dissection (ESD) has recently been attempted by expert endoscopists for *en bloc* resection of large sessile polyps[5,6]; however, this procedure has a long procedure time and high complication rate, and is not currently widely used due to its technical difficulty[7].

Several studies have shown that the incomplete removal of large sessile colorectal polyps, particularly by piecemeal resection, can contribute to a higher subsequent incidence of colorectal cancers[8,9]. Thus, recent guidelines recommend that when large sessile polyps are removed by piecemeal resection, a repeat examination should be performed at a short interval (2-6 mo) to verify complete removal[10]. However, few studies report the recurrence rate after EPMR, and the long-term outcomes of EPMR have yet to be established. This study was designed to evaluate the safety, efficacy, and long-term outcomes of EPMR of large sessile colorectal polyps.

MATERIALS AND METHODS

We retrospectively reviewed and identified 77 patients with 80 large sessile colorectal polyps (2 cm or greater) detected by colonoscopy at the National Cancer Center, Korea, between December 2002 and October 2005. Among these patients, 30 were excluded for the following reasons: co-existence of synchronous advanced colorectal cancer (n = 15), non-lifting tumor (n = 4), encircling lesion > 70% (n = 4), transfer to other institution (n = 4), suspicion of muscle invasion by endoscopic ultrasound (n = 1), and recurrent tumor (n = 2). A final total of 47 patients with 50 large sessile polyps who underwent EPMR using submucosal saline injection technique were enrolled in this study. We reviewed medical records, including patient demographics, endoscopic findings, histopathological reports, and follow-up data. The study was performed in accordance with the Declaration of Helsinki and informed consent was obtained from all patients.

Colonoscopy

Patients were prepared with mechanical bowel preparation. The patients received either two 45 mL doses of sodium phosphate (Fleet®; C.B. Fleet Co. Inc., Lynchburg, VA, USA) or 4 L of polyethylene glycol solution (Colyte-F®; Taejoon Pharm, Seoul, Korea) and underwent colonoscopy under conscious sedation with midazolam. Colonoscopy was performed to the cecum or terminal ileum with white light colonoscopic examination. Polyps suspicious of invasive cancer (the presence of ulceration, induration, friable mucosa, or non-lifting sign[11]) were referred for surgical resection. Polyp size (measured in comparison with open biopsy forceps) and morphology were generally estimated and recorded by the endoscopist.

EPMR technique

Endoscopic resection was performed using a snare piecemeal method with submucosal saline injection technique (Figure 1) according to the strip biopsy method described by Karita *et al*[12] Colonoscopy was performed with a standard video colonoscope (CF Q260L; Olympus Optical Co., Ltd., Tokyo, Japan). Briefly, the injection catheter was passed through the channel, and saline solution mixed with diluted epinephrine (1:100 000) was injected into the submucosal layer near the sessile polyp until the entire polyp was elevated. If the polyp was not elevated after one injection, additional injections were made around the polyp. The injection catheter was removed and a snare device was inserted through the channel. The surrounding normal mucosa, along with the lesion, was encircled by the snare. After the snare device was positioned, resection was performed using electrosurgical coagulation current, a combination of coagulation and blended currents in sequence. If necessary, argon plasma coagulation (APC) was used to treat the polyp base in an attempt to destroy any residual polyp.

Complications

EPMR-induced bleeding was defined as intraprocedural (occurring during EMR), early (within 24 h after EPMR), or delayed (≥ 24 h after EPMR), as described previously[13]. The diagnosis of early or delayed bleeding was based on the passage of blood per rectum. Bleeding was controlled by endoscopic clip (HX-600-135, Olympus) placement and/or APC. Perforation was defined as the presence of free air on plain abdominal film or computed tomography (CT).

Histopathology

All resected material was retrieved for histopathologic evaluation. Specimens were collected using a basket or by aspiration into the suction channel. For entire retrieval of sessile polyps located in the right colon, the colonoscope was withdrawn and reinserted as many times as necessary. A single pathologist assessed all histopathologic specimens and was not blinded to the endoscopic findings. Malignant polyps with unfavorable histology, such as poor differentiation, angiolymphatic invasion, and deep submucosal invasion (≥ 1000 μm), or having a positive deep resection margin, were referred for surgical treatment after polypectomy.

Follow-up

Patients who had undergone endoscopic treatment alone were followed up with colonoscopy to evaluate whether endoscopic resection of the sessile polyps had been complete. Endoscopic examinations were scheduled as follows: the first follow-up endoscopic examination was

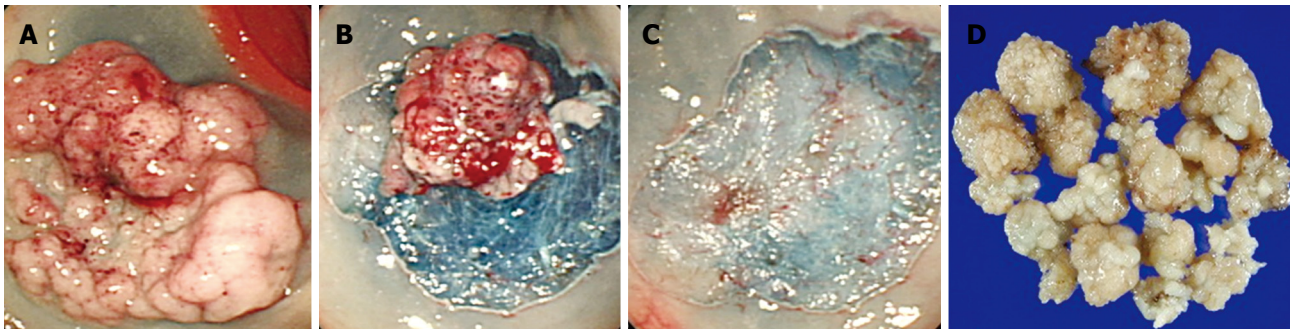


Figure 1 Endoscopic piecemeal mucosal resection of a large sessile polyp. A: Endoscopic view of the rectum showing a large sessile polyp (40 mm in diameter) after submucosal injection; B, C: Piecemeal polypectomy was performed; D: Retrieved specimens after resection of small pieces.

performed at 3-6 mo after the initial endoscopic resection, and the second was performed at 12 mo post-EPMR. Subsequent surveillance colonoscopic examinations were individualized with consideration of risk factors. If a polyp was detected on follow-up examinations, it was resected if possible. Residual polypoid tissue with the appearance of granulation tissue was biopsied, but not counted as residual adenoma. Recurrence or residual polyp was defined as the presence of any amount of adenomatous or carcinomatous tissue on follow-up, even as small as 1 mm, confirmed by histology at the site of prior piecemeal polypectomy[14]. All recurrences were demonstrated by pathology to contain dysplastic (adenoma) or carcinomatous tissue. The difference in recurrence between EPMR for benign polyps and EPMR for malignant polyps was determined by the log-rank test using SPSS 14.0 for Windows (SPSS Inc., Chicago, IL, USA). P values of < 0.05 were considered statistically significant.

RESULTS

The clinicopathologic characteristics of the 47 patients with 50 polyps are listed in Table 1. Among the patients, 5 had a history of non-steroidal anti-inflammatory drugs (NSAIDs) administration that was stopped at least 7 d before endoscopy. The mean size of resected polyps was 30.1 ± 10.9 mm (range, 20-60 mm). Locations of the large sessile polyps were as follows: cecum in 2 polyps, ascending in 7, hepatic flexure in 6, transverse in 4, splenic flexure in 1, descending in 5, sigmoid in 6, and rectum in 19. Of the 50 polyps, 34 (68%) were benign and 16 (32%) were malignant. Histological examination revealed tubular adenoma in 20 polyps, tubulovillous adenoma in 7, villous adenoma in 1, serrated adenoma in 4, hyperplastic polyp in 2, and carcinoma in 16 (Tis, 11; T1, 4; T2, 1).

The outcomes of EPMR for large sessile polyps are listed in Table 2. After EPMR, there were 6 (12%) cases of procedural bleeding: 5 intraprocedural and 1 early after initial endoscopic resection, which were managed by endoscopic means with the application of hemoclips alone in 4 cases, APC alone in 1 case, and hemoclips with APC plus fibrin glue in 1 case. There was no significant bleeding requiring blood transfusion or surgical intervention, and no delayed bleeding following polypectomy. No patient suffered colonic perforation as a result of endoscopic resection. Bleeding prophylaxis was performed in 6 cases (12%) by applying a hemoclip. The median hospital stay (from procedure to discharge) was 3 d (range, 1-5 d), with the exception of 4 patients: 3 who were not admitted and 1 who underwent consecutive colonic resection.

Table 1 Clinicopathologic characteristics of 47 patients with 50 large sessile polyps treated by endoscopic piecemeal mucosal resection

Variable	*n*
Male/female	23/24
Mean age (range), yr	60 (27-78)
Mean polyp size (range), mm	30.1 (20-60)
Location	
Cecum	2
Ascending	7
Hepatic flexure	6
Transverse	4
Splenic flexure	1
Descending	5
Sigmoid	6
Rectum	19
Histology	
Benign (n = 34)	
Tubular adenoma	20
Tubulovillous adenoma	7
Villous adenoma	1
Serrated adenoma	4
Hyperplastic	2
Malignant (n = 16)	
Tis	11
T1	4
T2	1

Of the 47 patients, follow-up data were available for 45 patients (95.7%); the exceptions were 2 without follow-up endoscopy. The median follow-up time was 37 mo (range, 3-72 mo). Recurrence was identified in 5 patients (12.2%): including 4 cases of local recurrence detected at 3 mo post-EPMR and 1 local recurrence detected at 14 mo post-EPMR (Table 3).

Of the 32 patients with benign polyps, there was 1 (3.1%) case of recurrence: a patient with benign recurrent adenoma who was successfully treated by endoscopic resection. Other follow-up endoscopy showed no recurrence. Of the 16 patients with malignant polyps, 4 polyps

Table 2 Clinical outcome of endoscopic piecemeal mucosal resection for large sessile polyps

Variable	*n*
Median follow-up[1] (range), mo	37 (3-72)
Median hospital stay[2] (range), d	3 (1-5)
Complications	
Bleeding (%)	6 (12)
Intraprocedural	5
Early	1
Delayed	0
Perforation	0
Recurrence (%)	5 (12.2)

[1]2 patients lost to follow-up were excluded; [2]Excluding 4 patients: 3 were not admitted and 1 underwent consecutive colonic resection.

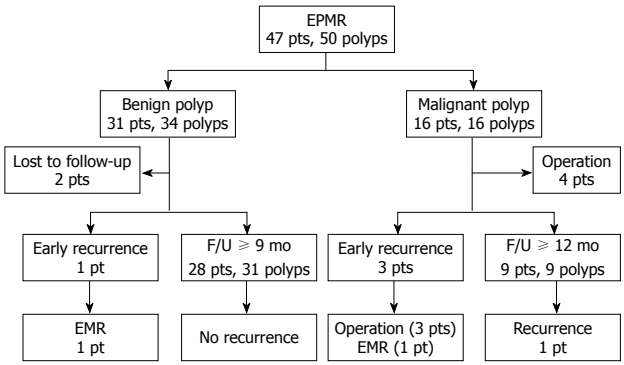


Figure 2 Long-term follow-up results for large sessile polyps. EPMR: Endoscopic piecemeal mucosal resection; F/U: Follow-up time; EMR: Endoscopic mucosal resection.

Table 3 Characteristics of 5 patients with residual/recurrent lesions after initial endoscopic piecemeal resection (EPMR)

No.	Age/sex	Location	Size (mm)	Primary histology	Time to recurrence (mo)	Recurrent histology	Method of treatment	Follow-up (mo)
1	72/M	Cecum	40	Adenoma	3	Adenoma	EMR	25
2	65/M	Rectum	40	Carcinoma (Tis)	3	Carcinoma (T2)	LAR	16
3	78/F	Rectum	40	Carcinoma (T1)	3	Adenoma	EMR	51
4	64/F	Rectum	60	Carcinoma (T1)	14	Carcinoma (Tis)	LAR	42
5	49/M	Sigmoid	40	Carcinoma (T1)	3	HGD adenoma	AR	40

HGD: High grade dysplasia; EMR: Endoscopic mucosal resection; LAR: Low anterior resection; AR: Anterior resection.

with unfavorable histology underwent surgery. Among the remaining 12 patients with malignant polyps, 4 (33.3%) had recurrence: 1 patient with benign recurrent adenoma was treated by endoscopic resection and 3 patients underwent surgery because their recurrent lesions could not be removed by endoscopic resection (Table 3).

The difference in recurrence between EPMR for benign polyps and that for malignant polyps was statistically significant (3.1% *vs* 33.3%, $P < 0.05$).

The follow-up results of the 50 large sessile polyps are shown in Figure 2.

DISCUSSION

Endoscopic resection of large sessile colorectal polyps (2 cm or greater) is increasingly used as an alternative to surgery, but remains challenging because of its technical difficulty, the high risk of complications such as bleeding or perforation, and the possibility of coexisting malignancy. Recent advances in endoscopic technique and equipment have enabled the development of techniques such as EPMR and ESD to remove large sessile colorectal polyps[1-6]. Comparing these two techniques, the mean procedure times for EPMR[4,6] and ESD[6,15] ranged from 29 to 55 min and from 70.5 to 108 min, respectively. Regarding complications, the perforation rates for EPMR[6,8,14] and ESD[4,6,15] ranged from 0.8% to 1.3% and 6.2% to 10.0%, respectively. Because EPMR has a shorter procedure time and lower perforation rate than ESD, EPMR appears to be an easy and safe procedure; however, Saito *et al*[6] demonstrated that EPMR resulted in a higher recurrence rate compared with ESD (14% *vs* 2%). The rate of recurrence at the polypectomy site after EPMR was reported to be 20%-55% in several other studies[8,14,16]. The recurrence rate after EPMR of 12.2% in the present study (11.36% in 44 polyps) is relatively low for the 44 large sessile polyps that had follow-up endoscopy. Several investigators have reported that additional techniques (e.g. APC) may further improve the success of polypectomy, and hence lower the recurrence rate[3,14,17]. In the present study, 91% of cases were treated with APC. Our low recurrence rate was probably influenced by the high application rate of APC to the tumor bed following EPMR. With regard to histopathology of the polyps, the recurrence rate was 3.1% for benign polyps and 33.3% for malignant polyps; in other words, EPMR of malignant polyps resulted in a higher recurrence rate than that of benign polyps. In contrast, Conio *et al*[13] reported similar recurrence rates for benign and malignant polyps. The recurrence rate of malignant colorectal polyps after EPMR varies among studies; however, it is difficult to compare the results of different series because there are wide variations in polyp size and the length of follow-up[13]. In addition, it is difficult to explain the reason why the incidence of recurrence after EPMR in malignant lesions is higher than that in benign lesions. However, we should try to remove all cancer cells completely because microscopic residual cancer cells after EPMR can cause recurrences. Further studies are needed to confirm the usefulness of EPMR for malignant colorectal polyps.

The most common complication after polypectomy is bleeding; the risk of post-polypectomy bleeding ranges from 0.3% to 6.1%[18,19]. The risk factors for bleeding include large polyp size and location in the proximal colon[19,20]. In large sessile polyps (2 cm or greater), the incidence of bleeding during and after polypectomy has been reported to be as high as 13.5%[2,3,8,14,16]. In the present study, bleeding occurred in 5 cases (12.2%), and all patients with bleeding were treated by endoscopic management, without surgery or blood transfusion. Thus, we can consider these cases as having "minor complications," as described in a previous report[3]. In the present study, none of the patients with bleeding were taking NSAIDs (aspirin) or had known coagulopathy at the time of the EPMR procedure. In our endoscopic database, any procedural bleeding requiring additional endoscopic treatment was described, and we classified EPMR-induced bleeding as intraprocedural, early, or delayed. Doniec *et al*[21] suggested that it is doubtful whether hemorrhage should be classified as a complication during endoscopic treatment when it can be managed endoscopically; no surgeon would regard bleeding as a complication during an operation such as mucosectomy. We used submucosal injection of epinephrine-saline mixture 1:100 000 in all EPMR cases, not only for submucosal elevation but also to prevent procedure-related bleeding. Iishi *et al*[2] injected only 0.9% saline solution alone to elevate sessile polyps, and reported bleeding in 7% of cases; however, no study has definitely proved the superiority of submucosal solution. In the present study, there was no delayed bleeding or perforations. Taking our experience into consideration, it is clear that the risk of perforation increases with increased width of the polypectomized colonic wall during snaring, rather than with increasing polyp size.

Rectal polyps are considered easy to remove due to the relatively low rate of perforation. In our study, 19 (40.4%) out of 47 polyps were located in the rectum. However, the recurrence rate after EPMR was not different between rectal polyps and colonic polyps (17.6% *vs* 7.4%, $P = 0.359$).

Previous studies have reported the risk of malignancy in large sessile colorectal polyps (2 cm or larger) as being up to 29%[22,23]. In the present study, 16 polyps (32%) were found to be adenomas containing an area of carcinoma; of these, 5 (10%) were invasive cancer. We evaluated the lifting sign of the tumor using saline injection to the submucosal layer before EPMR in all cases. This technique may have caused the relatively low incidence of invasive cancer in the large sessile colorectal polyps in the present study.

It is well known that initial colonoscopy has a significant miss rate of 24% for all types of adenomas[24]. Yamaji *et al*[25] reported recurrence rates for small adenomas and advanced lesions of 19.3% and 22.9%, respectively. In the present study, 80% of recurrences were identified at 3 mo post-EPMR, and the other recurrences were detected at 14 mo post-EPMR. Missed or metachronous adenomas detected at 3-6 mo, 1-3 years, and 3 years post-EPMR were detected in 32%, 46%, and 32% of cases, respectively. The higher rate of metachronous adenomas may have been influenced by the fact that our patients had more advanced lesions (i.e. all had large sessile polyps), including invasive cancers. The present results support current guidelines which recommend that patients who undergo piecemeal resection of large sessile adenomas should have an initial follow-up colonoscopy within 3-6 mo, followed by an additional colonoscopy 1 year later[10].

In conclusion, EPMR is a safe procedure for large sessile colorectal polyps, but should be applied carefully in malignant polyps because of high recurrence rate. Close follow-up endoscopic examinations are necessary for early detection of recurrence.

COMMENTS

Background

Endoscopic removal of colonic adenomatous polyps is a commonly used technique that reduces the incidence of colorectal cancer. However, endoscopic resection of large sessile colorectal polyps is still challenging because of its technical difficulty.

Research frontiers

Endoscopic piecemeal mucosal resection (EPMR) can be used for large sessile colorectal polyps. However, this approach remains controversial because of the high possibility of coexisting malignancy and the high recurrence rate associated with large sessile polyps. Few studies report the recurrence rate after EPMR, and the long-term outcomes of EPMR have yet to be established.

Innovations and breakthroughs

Several studies have shown that the incomplete removal of large sessile colorectal polyps, particularly by piecemeal resection, can contribute to a higher subsequent incidence of colorectal cancers. This study shows that EPMR is safe for benign colorectal polyps, but should be applied carefully in malignant polyps due to high recurrence rate.

Applications

By identifying the recurrence rate and long term follow-up results after EPMR, this study may contribute to the development of a therapeutic guideline for patients with large sessile colorectal polyps.

Terminology

EPMR, which is used to increase safety, is a technique for removing large sessile polyps. During EPMR, the polyp can be broken down into multiple pieces. However, this makes it difficult to evaluate the resection margin pathologically.

Peer review

This paper is devoted to obtaining knowledge on the outcome of piecemeal mucosal and large sessile polyps. The authors have concluded that EPMR should be carefully applied in malignant polyps.

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BRIEF ARTICLE

Gastrointestinal symptoms in patients undergoing peritoneal dialysis: Multivariate analysis of correlated factors

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Abstract

AIM: To investigate gastrointestinal (GI) symptoms in peritoneal dialysis (PD) patients and to explore related factors contributing to GI symptoms.

METHODS: One hundred and twelve patients undergoing PD participated in the study. The gastrointestinal symptom rating scale was used for measuring GI symptoms. Information on age, height, weight, body mass index, disease leading to chronic renal failure, history of corticosteroid therapy, presence of predialytic GI symptoms, daily dosage of pills, and duration, type and daily dialysate volume of PD was obtained by interviewing patients and/or reviewing the medical records. Hemoglobin, albumin and Kt/V data were obtained from follow-up database. We used multiple regression analysis with stepwise backward variable selection to test for factors predicting GSRS scores with significance level of selection entry at 0.05 and selection of stay at 0.10.

RESULTS: The prevalence of eating dysfunction, reflux and indigestion in the PD patients was 44.2%, 32.7%, 32.7%, respectively. A history of corticosteroid therapy (b = 8.93, $P < 0.001$) and all pills daily intake (b = 0.16, $P = 0.007$) were positively correlated to GI symptoms, while residual renal Kt/V (b = -3.47, $P = 0.009$) was negatively correlated to GI symptoms. Other factors were proven to be not associated with GI symptoms, with $P > 0.05$.

CONCLUSION: Eating dysfunction, reflux and indigestion were common in PD patients. Daily dosage of pills and corticosteroid history predicted GI symptoms, while residual renal function prevented them.

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Key words: Eating dysfunction; Gastroesophageal reflux; Dyspepsia; Peritoneal dialysis; Residual renal function

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INTRODUCTION

Gastrointestinal (GI) symptoms are common in patients with chronic renal failure (CRF)[1-3], especially in patients having continuous ambulatory peritoneal dialysis (CAPD)[4]. A variety of GI symptoms in CAPD patients have been reported[4,5], of which, gastroesophageal reflux symptoms (GERS), dyspepsia and eating dysfunction seem to be the most common ones[4,5].

Anderson *et al*[6] reported that 44.7% of PD patients

had frequent gastroesophageal reflux disease (GERD) and that age < 60 years, smoking, and body mass index (BMI) ⩾ 27 predicted GERD; in contrast, sex, race, diabetes, PD, non steroidal anti-inflammatory drugs (NSAIDs), calcium channel blockers (CCBs), and coffee and alcohol use did not. Stojakowska *et al*[7] have proven a negative correlation between GERD symptom score index and normalized protein catabolic rate (nPCR), and a positive correlation between GERD symptom score and the time from onset of CAPD, through observation in 43 patients. It is not clear whether PD *per se* is a risk factor for GERD[6].

It is the high prevalence of GI symptoms in PD patients that raises questions about contributing factors and other possible factors, but previous studies obtained controversial results with a relatively small sample size. Whether the onset of these GI symptoms is related to the chronic renal failure itself, its treatment, or, alternatively, other factors, is still unknown.

The aim of this study was to investigate GI symptoms in CAPD patients and to explore the possible correlated factors contributing to these symptoms.

MATERIALS AND METHODS

Ethics

Patients gave informed consent and the study was approved by the Ethics Committee of Changhai Hospital, Shanghai, China.

Participants

The patients on active PD were recruited from the PD unit in Changhai Hospital. They consisted of in-patients and out-patients who maintained PD for at least three months. Patients with dementia, severe infectious illness, hepatocholecystopathy, peritonitis in the last three months, unstable blood pressure or glucose levels, and unwillingness to participate in the study were excluded.

Subjective gastrointestinal symptoms: the gastrointestinal symptom rating scale

To evaluate the presence of GI symptoms in PD patients, they were asked to complete the gastrointestinal symptom rating scale (GSRS) measuring GI symptoms in general.

The GSRS, a self-administered questionnaire, includes 15 items and uses a 7-grade Likert scale defined by descriptive anchors such that 1 = none, 2 = minor, 3 = mild, 4 = moderate, 5 = moderately severe, 6 = severe, and 7 = very severe discomfort. The questionnaire was originally constructed as an interview-based rating scale designed to evaluate a wide range of GI symptoms[8] and was later modified to become a self-administered questionnaire[9]. The items can be grouped into five dimensions: abdominal pain syndrome (three items), reflux syndrome (two items), indigestion syndrome (four items), diarrhea syndrome (three items), and constipation syndrome (three items). One dimension, eating dysfunction, which was developed in a manner analogous to the GSRS[10], was also considered relevant for the study and added to the original GSRS. Eating dysfunction dimension includes questions concerning early satiety, difficulties in eating normal portions, and postprandial pain. The questions concern symptom severity during the previous two weeks. A dimension score was calculated as the mean value of the items belonging to the specific syndrome with a minimum value of 1 and a maximum value of 7.

Patient information

By interviewing patients and/or reviewing the medical records we obtained information on age, height, weight, BMI, disease leading to CRF, history of corticosteroid therapy, presence of predialytic GI symptoms, daily dosage of pills and duration, type and daily dialysate volume of PD. The latest serum hemoglobin (HGB), albumin (ALB), and Kt/V urea, as an index of dialysis adequacy, were obtained from the follow-up database. Kt/V were calculated by Daugirdas Formula[11].

Statistical analysis

Data were presented as mean (SD) for continuous variables that were approximately normally distributed, as median and interquartile range for skewed continuous variables, and as percentage for categorical variables. We used multiple regression analysis with stepwise variable selection to test for factors that predicted the GSRS scores with significance level of selection entry at 0.05 and selection of stay at 0.10. Results were considered significant when $P < 0.05$. All analyses were performed with SPSS for Windows, version 16.0.

RESULTS

Patient characteristics

In total, one hundred and twelve PD patients were approached to participate in the study and completed the questionnaires. Table 1 presents characteristics of the included PD patients. The sex distribution among PD patients was 61 men and 51 women. The mean age among PD patients was 59.67 (14.18) years and the mean BMI was 23.26 (4.27) kg/m^2. Patients with diabetes mellitus (DM) made up 24.1% of the study population in total and a majority (87.5%) of PD patients had no GI symptoms before the start of PD. The median duration of PD was 15.00 (8.00-33.00) mo and 53.6% of the patients underwent CAPD.

The gastrointestinal symptom rating scale scores

The prevalence of troublesome GI symptoms (GSRS > 1) was 61.6% for any dimension, 44.2% for eating dysfunction, 32.7% for reflux, 32.7% for indigestion, 18.6% for constipation, 6.2% for abdominal pain, and 5.3% for diarrhea (Figure 1). The mean GSRS scores for eating dysfunction were 1.57 (0.84), for reflux 1.71 (1.15), for indigestion 1.32 (0.56), for constipation 1.23 (0.58), for diarrhea 1.07 (0.35), for abdominal pain 1.04 (0.19).

GSRS scores and patient variables

There was no significant relationship between the GSRS scores and age, BMI, hemoglobin, albumin, presence of

Table 1 Clinical features of the study population *n* (%)

Clinical features	Patient (*n* = 112)
Age (mean ± SD) (yr)	59.67 ± 14.18
Sex	
Male	61 (54.5)
Female	51 (45.5)
BMI, mean (SD) (kg/m^2)	23.26 (4.27)
DM status	
DM	27 (24.1)
Non-DM	85 (75.9)
Disease leading to chronic renal failure	
Chronic glomerulonephritis	40 (35.7)
Primary hypertension	31 (27.7)
Diabetes mellitus	22 (19.6)
Polycystic kidney disease	7 (6.2)
Gout	3 (2.7)
Primary hypertension combined with gout	3 (2.7)
Obstructive nephropathy	2 (1.8)
Chronic interstitial nephritis	1 (0.9)
Nephrotic syndrome	1 (0.9)
Ischemic renal disease	1 (0.9)
Microscopic Polyarteritis	1 (0.9)
Steroid history	
Yes	8 (7.1)
No	104 (92.9)
Daily dosage of pills, median (interquartile ranges)[1]	12.00 (6.00-21.25)
Predialytic GI symptoms	
Yes	14 (12.5)
No	98 (87.5)
PD Duration, median (interquartile ranges) (mo)[2]	15.00 (8.00-33.00)
Type of PD	
CAPD	60 (53.6)
IPD	50 (44.6)
Daily peritoneal dialysate volume (mean ± SD) (L)[3]	7.83 ± 1.28
Albumin (mean ± SD) (g/L)[4]	33.96 ± 4.77
Hemoglobin (mean ± SD) (g/L)[5]	101.24 ± 20.06
Residual renal Kt/V, median (interquartile ranges)[6]	0.39 (0.00-0.82)
Peritoneal Kt/V (mean ± SD)[6]	1.51 ± 0.39
Total Kt/V (mean ± SD)[6]	2.00 ± 0.51

[1]Restricted to the 106 patients (94.64% of the overall sample) for whom complete information on the daily dosage of pills was available; [2]Restricted to the 109 patients (97.32% of the overall sample) for whom complete information on peritoneal dialysis duration was available; [3]Restricted to the 110 patients (98.21% of the overall sample) for whom complete information on peritoneal dialysate volume was available. [4]Restricted to the 108 patients (96.43% of the overall sample) for whom complete information on serum albumin was available; [5]Restricted to the 110 patients (98.21% of the overall sample) for whom complete information on serum hemoglobin was available; [6]Restricted to the 81 patients (72.32% of the overall sample) for whom complete information on residual renal Kt/V, peritoneal Kt/V, and total Kt/V was available. BMI: Body mass index; DM: Diabetes mellitus; CAPD: Continuous ambulatory peritoneal dialysis; IPD: Intermittent peritoneal dialysis; GI: Gastrointestinal; PD: Peritoneal dialysis.

diabetes, presence of predialytic GI symptoms, peritoneal Kt/V, and duration, type and daily dialysate volume of PD, with $P > 0.05$ (Table 2).

There were three statistically significant predictors for the GSRS scores (Table 3). A history of corticosteroid therapy (b = 8.93, $P < 0.001$) was significantly related to the GSRS scores and its coefficient was positive, indicating that if a patient had a history of corticosteroid therapy, he/she would attain higher GSRS scores than a patient who had not. Next, the daily dosage of pills as patients

Table 2 Multiple stepwise regression of the variables not associated with the GSRS scores[1]

Variables	*t*	*P*-value
Age	1.96	0.54
DM status	1.01	0.31
BMI	-0.50	0.62
Hemoglobin	0.82	0.42
Albumin	0.68	0.50
Predialytic GI symptoms	0.49	0.62
PD type	-0.89	0.38
Duration of PD	-0.58	0.57
Daily dialysate volume	-1.15	0.26
Peritoneal Kt/V	-0.21	0.84
Total Kt/V	-0.24	0.81

[1]Restricted to the 81 patients (72.32% of the overall sample) for whom complete information on age, diabetes status, weight, height, serum hemoglobin, albumin levels, presence of predialytic gastrointestinal symptom, peritoneal dialysis type, duration of peritoneal dialysis, daily dialysate volume, history of corticosteroid use, daily dosage of pills, residual renal Kt/V, peritoneal Kt/V, total Kt/V were available. Adjusted for diabetic status (yes = 1, no = 0), presence of predialytic GI symptoms (yes = 1, no = 0), PD type (CAPD = 1, IPD = 0). GSRS: Gastrointestinal symptom rating scale.

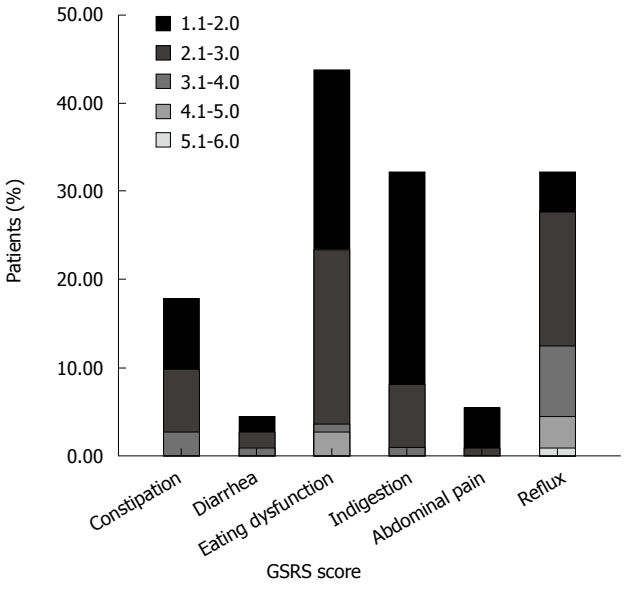


Figure 1 Prevalence and grading of gastrointestinal symptoms in peritoneal dialysis patients according to the gastrointestinal symptom rating scale.

daily intake (b = 0.16, $P = 0.007$) was significant and its coefficient was also positive, indicating that the more pills patients took daily, the higher the GSRS scores. Finally, the residual renal Kt/V (b = -3.47, $P = 0.009$) was significant and its coefficient was negative which would indicate that higher residual renal Kt/V was related to lower GSRS scores (Table 3).

DISCUSSION

The present study shows that gastrointestinal symptoms are common in PD patients, especially eating dysfunction (44.2%), reflux and indigestion (both 32.7%). A history of

Table 3 Coefficients of multiple stepwise regression model of the variables associated with GSRS scores[1]

Variables	Unstandardized coefficients		Standardized coefficients	*t*	*P* value
	B	SD	β		
Constant	21.89	1.25	-	17.58	< 0.001
History of corticosteroids	8.93	2.13	0.39	4.20	< 0.001
Daily dosage of pills	0.16	0.06	0.26	2.80	0.007
Residual renal Kt/V	-3.47	1.30	-0.25	-2.68	0.009

[1]Restricted to the 81 patients (72.32% of the overall sample) for whom complete information on age, diabetes status, weight, height, serum hemoglobin, albumin levels, presence of predialytic gastrointestinal symptoms, peritoneal dialysis type, duration of peritoneal dialysis, daily dialysate volume, history of corticosteroid use, daily dosage of pills, residual renal Kt/V, peritoneal Kt/V, total Kt/V were available. Adjusted for history of corticosteroid use (yes = 1, no = 0).

corticosteroid therapy (b = 8.93, $P < 0.001$) and the dosage of pills patients took daily (b = 0.16, $P = 0.007$) were positively related to GSRS scores, whereas residual renal Kt/V (b = -3.47, $P = 0.009$) was negatively correlated to GSRS scores. Other suspected factors were not statistically related to GSRS scores, including age, BMI, hemoglobin, albumin, presence of diabetes, presence of predialytic GI symptoms, peritoneal Kt/V, total Kt/V and duration, type and daily dialysate volume of PD.

More than half of the PD patients had various gastrointestinal complaints and patients who had a history of corticosteroid therapy seemed to be susceptible to gastrointestinal symptoms. The more pills patients took daily, the more complaints of GI symptoms. Otherwise, residual renal function seemed to be a protective factor for GI symptoms in PD patients since the higher the residual renal Kt/V, the less GI symptom complaints. In this study, PD specific factors including peritoneal Kt/V, total Kt/V, duration, type and daily dialysate volume of PD, were proven to contribute little to these symptoms.

GI symptoms are common in PD patients with a prevalence ranging from 43% to 58%[5-7,12]. Our results with a high prevalence of eating dysfunction, indigestion and reflux symptoms are in line with these previous studies. Furthermore, these three symptoms were also shown to have the highest prevalence of all GI symptoms in PD patients in a recent study[5]. The underlying pathophysiological mechanisms might be (1) Delayed gastric emptying, which is common in CRF patients. Strid *et al*[13] found that PD patients had longer gastric emptying time than predialytic state, but other studies found no obvious effect of dialysate on gastric emptying[14-16]; (2) Increased intraperitoneal pressure (IPP). Dejardin *et al*[17] found the occurrence of GERS was not different for patients with elevated day and night IPP; (3) Decreased lower esophageal sphincter pressure (LESP). Kim *et al*[18] demonstrated that CAPD patients with upper GI symptoms had lower LESP at 2000 mL of infused dialysate than patients without. In contrast, Hylander *et al*[19] found no systematic changes in intragastric or LESPs at any time of CAPD; and (4) Other factors. Aguilera *et al*[20] discovered GI abnormalities were negatively associated with nutrition. Van *et al*[21] have demonstrated a glucose-based dialysate in the abdomen of PD patients with delayed gastric emptying.

As we know, PD patients are in a complicated clinical condition and clinical manifestations are affected by many factors, among which drug taking is an important one. However, drugs have some adverse effects. In general, patients who have a history of corticosteroid therapy are more susceptible to GI symptoms than patients who haven't, which also applies to PD patients. Not unexpectedly, we found corticosteroid taking history alone accounted for 18.9% of GSRS scores, which is probably due to injury of the digestive system caused by corticosteroids. High prevalence of GI symptoms was also found in a transplant population and a rheumatism population[22,23], who both had a large consumption of corticosteroids.

Besides drug history, the daily dosage of pills patients take also plays an important role in causing GI symptoms. To our knowledge, this is the first study counting pills of patients' daily intake and looking for an association between the amount of pills and GI symptoms. We did find that the more pills taken, the more severe the GI symptoms. In our study, the average amount of pills a PD patient took daily was almost 15, and the maximum amount was 51, which consisted of CCBs, angiotensin converting enzyme inhibitors (ACEIs), angiotensin receptor blockers (ARBs), statins, active vitamin D, iron agents, ketoacids, anti-platelet drugs and varieties of Chinese medicine, which were especially common in China. The underlying mechanisms are not fully understood.

Compared to hemodialysis (HD), PD is thought to provide better preservation of residual renal function (RRF), which in many studies partly accounts for reduction of mortality risk, decreasing inflammation factors, declining oxidative and carbonyl stress[24-27]. In addition, in our study, another highlight of RRF we discovered was that it can prevent PD patients from developing GI symptoms. Apart from residual renal Kt/V, peritoneal Kt/V and total Kt/V were not proven to be correlated to GI symptom scores. These results indicated that the beneficial effect of residual renal clearance and peritoneal clearance were not equivalent with regard to patient outcome, and residual renal function may play a much more important role. Previous study has also shown that for each mL/min per 1.73 m^2 increase in residual renal glomerular filtration rate (GFR), a 12% reduction in mortality rate was found[26]. In contrast, no significant effect of peritoneal clearance on patient survival was established[26]. Regarding this, GI symptoms in PD patients were more associated with the uremia itself, therefore PD specific factors were not involved.

Previous study has concluded that age < 60 and BMI ⩾ 27 predicted GERD in the general population, while diabetes and PD did not[6]. However, we found neither age, BMI nor diabetes with PD predicted GI symptoms in PD patients. This may be due to the limitation in sample

size. Furthermore, albumin, as an index of nutrition, and duration of PD were not related to GI symptoms in our study. However, other studies obtained controversial results. A negative correlation between GERD symptom score and nPCR, and a positive correlation between GERD symptom score and the duration of CAPD, were found through observation in 43 patients[7]. Moreover, analyses of 99 dialytic patients revealed that gastrointestinal symptom scores were not different in hypoalbuminemic and normoalbuminemic patients[28]. In addition, gastrointestinal life quality was found not to be correlated with the duration of PD treatment[29]. Factors which contributed to these conflicting results might be the sample size and the different ways used in evaluating the GI symptoms.

Our study included only CAPD and intermittent peritoneal dialysis (IPD) patients and neither of these groups was independently associated with GI symptom scores. Further evaluation in a larger population with different types of PD is needed for finding out the relation between PD type and GI symptoms.

Volume of dialysis fluid used was also a non-predictor. Another study recruited 61 PD patients and showed a strong linear correlation between IPP and intraperitoneal volume, but failed to find any influence of IPP on the occurrence of GERS except that patients with GERS had a higher BMI[17]. The increased IPP was still not a rational explanation for the high prevalence of GI symptoms in the PD population.

One limitation of the present study is the relatively small number of samples, which may influence the power of statistical tests. Some factors, such as age, daily dosage and PD type showed a tendency to be correlated with GI symptoms, but correlation did not reach statistical significance because of the fact that 31 of 112 samples were excluded from the multiple regression model for the missing value of Kt/V and we only recruited patients with CAPD and IPD. Another limitation refers to the probable subjective bias in answering the questionnaire. Only one self-administered questionnaire was used in the study. Still another limitation refers to the lack of a control group consisting of patients undergoing HD.

In conclusion, the present study demonstrates a high prevalence of troublesome GI symptoms in PD patients, a positive correlation between history of corticosteroid use, the amount of pills patients take daily, and GI symptoms, and a negative correlation between residual renal function and GI symptoms. GI symptoms are more common in PD than in HD patients as well as compared to the predialytic population[4] which strongly suggests that PD treatment is a putative cause of GI symptoms. However, the present study failed to prove a relationship between PD specific factors and GI symptoms. Therefore, further evaluation in a larger population of PD patients with a control group is needed.

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COMMENTS

Background

Gastrointestinal (GI) symptoms are more common in patients undergoing peritoneal dialysis (PD) than in patients with chronic renal failure undergoing hemodialysis, though the cause and the correlated factors are largely unknown. Uremia itself and the impaired digestive system caused by PD are the main suspected causes.

Research frontiers

PD patients are proven to have a higher prevalence of GI symptoms. Many studies have focused on the impact of dialysate on the gastrointestinal tract but obtained controversial results. In this study, the authors demonstrate that gastrointestinal complaints in PD patients are more related to a history of corticosteroid therapy, the number of pills taken daily and residual renal function rather than effects of the dialysate.

Innovations and breakthroughs

Recent reports have highlighted the high prevalence of GI symptoms in PD patients and their poor treatment. This is the first study exploring possible correlated factors from almost all details in the life of PD patients. In addition, this is the first study demonstrating that dialysate is not the main cause of GI symptoms in PD patients. Furthermore, this study suggests that GI symptoms in PD patients may be affected by residual renal function and drug history.

Applications

By understanding what factors are related to GI symptoms in PD patients, this study may represent a future strategy for prevention and intervention in the treatment of PD patients with GI symptoms.

Terminology

GSRS, Gastrointestinal Symptom Rating Scale, a self-administered questionnaire, includes 15 items and uses a 7-grade Likert scale defined by descriptive anchors such that 1 = none, 2 = minor, 3 = mild, 4 = moderate, 5 = moderately severe, 6 = severe, and 7 = very severe discomfort. The items can be grouped into five dimensions: abdominal pain syndrome (three items), reflux syndrome (two items), indigestion syndrome (four items), diarrhea syndrome (three items), and constipation syndrome (three items). One dimension, eating dysfunction, which was developed in a manner analogous to the GSRS, was also considered relevant for the study and added to the original GSRS.

Peer review

This is a straightforward, well done study with straightforward conclusions. I hope the authors will take the next step and perform a more rigorous study with 2 control arms (chronic renal failure and HD) in a prospective fashion.

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BRIEF ARTICLE

Laparoscopic *vs* open left hepatectomy for hepatolithiasis

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Abstract

AIM: To explore the feasibility and therapeutic effect of total laparoscopic left hepatectomy (LLH) for hepatolithiasis.

METHODS: From June 2006 to October 2009, 61 consecutive patients with hepatolithiasis who met the inclusion criteria for LLH were treated in our institute. Of the 61 patients with hepatolithiasis, 28 underwent LLH (LLH group) and 33 underwent open left hepatectomy (OLH group). Clinical data including operation time, intraoperative blood loss, postoperative complication rate, postoperative hospital stay time, stone clearance and recurrence rate were retrospectively analyzed and compared between the two groups.

RESULTS: LLH was successfully performed in 28 patients. The operation time of LLH group was longer than that of OLH group (158 ± 43 min *vs* 132 ± 39 min, $P < 0.05$) and the hospital stay time of LLH group was shorter than that of OLH group (6.8 ± 2.8 d *vs* 10.2 ± 3.4 d, $P < 0.01$). No difference was found in intraoperative blood loss (180 ± 56 mL *vs* 184 ± 50 mL), postoperative complication rate (14.2% *vs* 15.2%), and stone residual rate (intermediate rate 17.9% *vs* 12.1% and final rate 0% *vs* 0%) between the two groups. No perioperative death occurred in either group. Fifty-seven patients (93.4%) were followed up for 2-40 mo (mean 17 mo), including 27 in LLH group and 30 in OLH group. Stone recurrence occurred in 1 patient of each group.

CONCLUSION: LLH for hepatolithiasis is feasible and safe in selected patients with an equal therapeutic effect to that of traditional open hepatectomy.

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Key words: Hepatolithiasis; Laparoscopy; Hepatectomy; Complication; Therapeutic effect

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INTRODUCTION

Hepatolithiasis refers to the stone in branching bile ducts above the confluence of left and right hepatic ducts. It may occur alone or with extrahepatic bile duct stones and is a prevalent disease in Southeast Asia and its incidence is also higher in Chinese coast areas, southwest region, Hong Kong and Taiwan. Hepatectomy is a definite and effective approach for hepatolithiasis[1,2]. With the refinement of laparoscopic instruments and accumulated experience in both laparoscopic surgery and hepatic surgery, laparoscopic hepatectomy has been used in treatment of hepatic benign and malignant tumors and donor hepatectomy of live donor liver transplantation[3,4]. However, few studies are available on laparoscopic hepatectomy for hepatolithiasis[5-8]. We successfully used total laparoscopic left hepatectomy (LLH) to treat hepatolithiasis in

8 patients between November 2003 and May 2006, and have preliminarily accumulated surgical experience with it and considered this operation safe and feasible[9]. To further explore the therapeutic effect of total LLH on hepatolithiasis, clinical data about 61 consecutive patients with hepatolithiasis who underwent LLH or open left hepatectomy (OLH) were retrospectively analyzed and compared.

MATERIALS AND METHODS

Inclusion criteria and patients

The inclusion criteria for LLH for hepatolithiasis include (1) multiple stones in the left or left lateral intrahepatic ducts with fibrosis and atrophy of hepatic lobes or hepatic segments; (2) possibly combined with extrahepatic bile duct stones or a few stones in the right intrahepatic ducts, but with no extrahepatic bile duct stricture or stone incarceration in the lower part of common bile duct; and (3) liver function of Child A to B classification, with no portal hypertension, coagulation disorder, structural disease in the heart, lungs, liver and kidneys, intrahepatic biliary cancer, and abdominal surgical histories.

From June 2006 to October 2009, 61 consecutive patients with hepatolithiasis who met the inclusion criteria for LLH were treated in our institute. Of the 61 patients with hepatolithiasis, 28 underwent LLH (LLH group) and 33 underwent OLH (OLH group). Before operation, all patients had a complete medical evaluation, including liver function, renal function, electrocardiogram and chest X-ray. Preoperative ultrasonography, CT and MRCP were performed to identify the distribution of stones and changes in the bile duct tree. Of the 28 patients in LLH group, 10 were men and 18 women, with a mean age of 47 years (range 25-63 years). Twenty-one patients had left hepatolithiasis and 7 left and right hepatolithiasis. Twelve patients were accompanied with cholecystolithiasis, 13 with choledocholith, and 6 with mild jaundice. Liver function was classified as Child A and B in 22 and 6 patients, respectively. Two patients had a history of biliary surgery. Of the 33 patients in OLH group, 12 were men and 21 women with a mean age of 49 years (range 31-68 years). Twenty-three patients had left hepatolithiasis and 10 left and right hepatolithiasis. Twelve patients were accompanied with cholecystolithiasis, 15 with choledocholithiasis, and 9 with mild jaundice. Liver function was classified as Child A and B in 25 and 8 patients, respectively. Three patients had a history of biliary surgery. No significant difference was found in age, sex, stone distribution, liver function and surgical history between the two groups.

Operative techniques

Patients in LLH group, placed in supine position, underwent general anesthesia. The chief surgeon stood on the left of the patient and the first assistant on the right. Another surgeon who supported the mirror stood on the left of the chief surgeon. Two monitors were placed above each side of the patient's head. A 10-mm cut was made below the umbilicus and a CO_2 pneumoperitoneum was established at a pressure of 14 mmHg. A 30° angled laparoscope was introduced. Under direct vision, two 12-mm trocars were respectively inserted below the xiphoid bone and the costal margin of the left mid-clavicular line, and a 5-mm trocar was also inserted below the costal margin of the right mid-clavicular line.

Left lateral segmentectomy: Laparoscopy was performed with the round and falciform ligaments transected using an ultrasound knife (Johnson and Johnson, USA). By meticulous dissection, the artery and vein of left lateral segment were visualized, clamped with absorbable clips and divided. Interrupted bile ducts were not clamped temporarily. Left triangular and coronary ligaments were divided with the trunk or branch of the left hepatic vein carefully dissected properly away from the second hepatic portal. If the confluence point of left hepatic veins and inferior vena cava was very close to the posterior border of the left liver, the left hepatic veins were clamped extremely near the posterior border of the left liver, but not divided until complete removal of Couinaud. If the confluence point of left hepatic veins and inferior vena cava was away from the liver parenchyma, the left hepatic vein was dissected, clamped with absorbable clips and divided (Figure 1). Liver parenchyma was transected from left of the round ligament to liver inferior border of sagittal portion, vessels and bile ducts in the transection plane were bluntly dissected, clamped and divided. Dilated intrahepatic bile ducts in the transection plane were opened. According to the size, number and position of residual stones, stone forceps were introduced below the xiphoid bone to directly remove stones, or a fiber choledochoscope (Olympus, Japan) was used to remove stones from the transection plane. If the stones were near the distal common bile duct or bigger, or combined with right hepatolithiasis, common bile duct exploration was performed to remove the stones. Cholecystectomy was done routinely. Continuous or interrupted suture was performed for the transection plane with 3-0 Vicryl. Water was injected *via* a T tube to determine whether the suture was tight. The transection plane was coagulated with fibrin glue to seal capillary vessels or covered with absorbable hemostatic gauze. The integral specimen was packed into a plastic bag and removed *via* an extended trocar hole. A drainage tube was left in vicinity of the transection plane through the costal margin of the left mid-clavicular line. Whether a drainage tube is left near the first hepatic portal depends on the intraoperative conditions.

Left hemihepatectomy: In the first hepatic portal, the left hepatic artery and the left branch of portal vein were dissected. The proximal end of left hepatic artery was clamped with two absorbable clips, the left hepatic artery was clamped with a mental soft clip 2-3 mm away from the absorbable clips, and the left hepatic artery was divided between mental and absorbable clips. The left branch of portal vein was treated with the same method

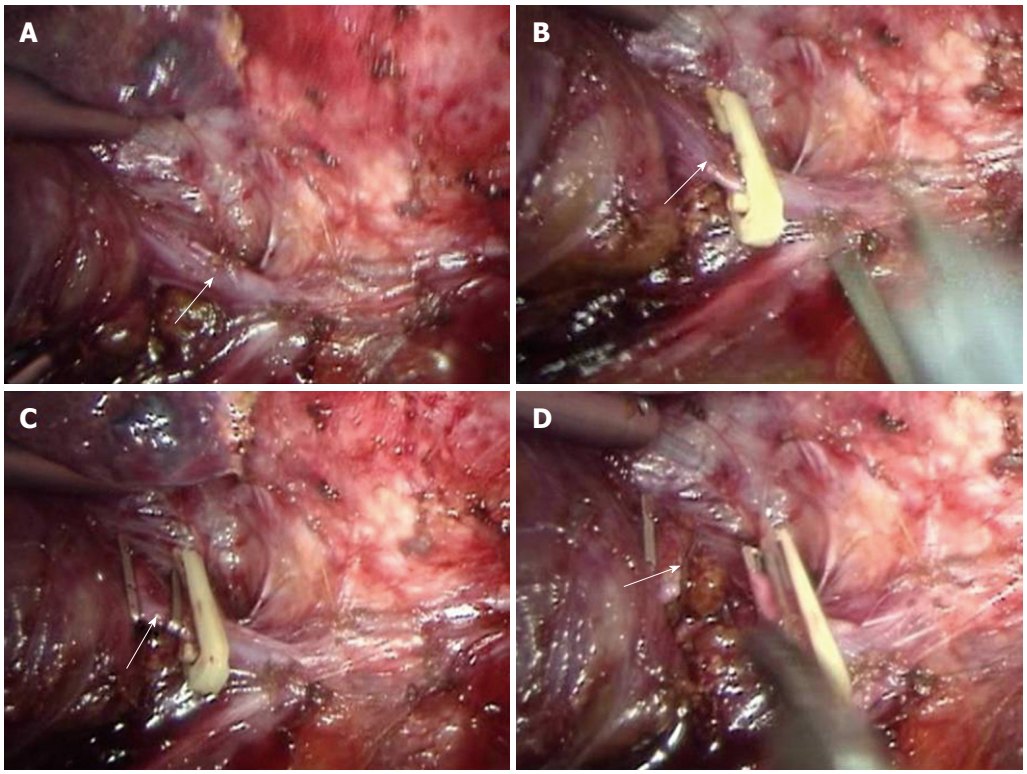


Figure 1 Laparoscopy showing dissected LHV (A, arrow), clamped LHV (B and C, arrow), and transected LHV (D, arrow). LHV: Left hepatic vein.

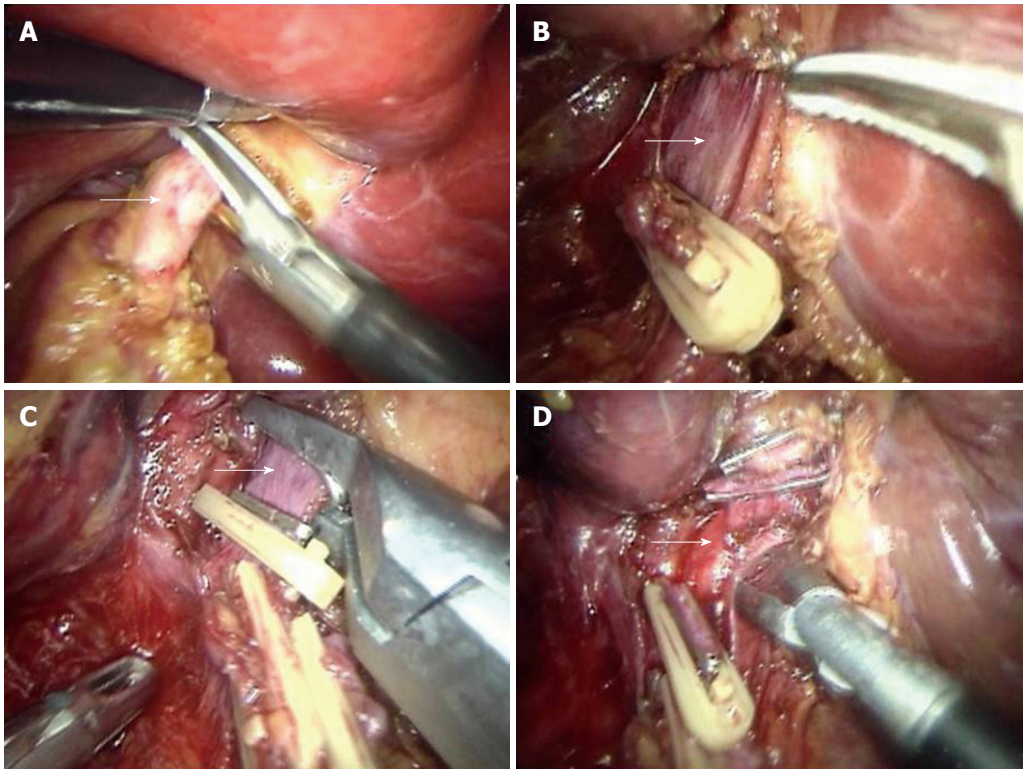


Figure 2 Laparoscopy showing dissected LBHA (A, arrow), exposed LBHV (B, arrow), dissected and clamped LBHV (C, arrow), and dissected left hepatic duct (D, arrow). LBHA: Left branch of hepatic artery; LBHV: Left branch of hepatic vein.

(Figure 2). Other surgical procedures were the same as those in left lateral segmentectomy.

In OLH group, an oblique incision was made along the right costal margin or along the right rectus abdominis with the patients in supine position under general anesthesia. Cholecystectomy was done routinely. Common

Table 1 Surgical procedure for hepatolithiasis in 61 patients

Variables	LLH (n = 28)	OLH (n = 33)
Left hemihepatectomy	3	5
Left lateral segmentectomy	25	28
Combined with cholecystectomy	26	30
Combined with CBD exploration	22	31
T tube drainage	16	27
Primary suture of CBD	6	4
Intraoperative choledochoscope	28	28

LLH: Laparoscopic left hepatectomy; OLH: Open left hepatectomy; CBD: Common bile duct.

Table 2 Comparison between LLH and OLH for hepatolithiasis n (%)

Variables	LLH (n = 28)	OLH (n = 33)
Operating time (min)	158 ± 43	132 ± 39
Intraoperative blood loss (mL)	180 ± 56	184 ± 50
Intraoperative blood transfusion	0 (0)	1 (3.0)
Postoperative complications	4 (14.2)	5 (15.2)
Postoperative hospital stay (d)	6.8 ± 2.8	10.2 ± 3.4
Intermediate residual stone	5 (17.9)	4 (12.1)
Final residual stone	0 (0)	0 (0)
Stone recurrence	1 (3.6)	1 (3.0)
Perioperative mortality	0 (0)	0 (0)

bile duct exploration was performed to remove stones. Couinaud was removed with a T drainage tube left.

Residual stones were completely removed by fiber choledochoscopy *via* the T tube sinus tract after operation in both groups.

Clinical data including operation time, intraoperative blood loss, postoperative complication rate, postoperative hospital stay time, and stone clearance and recurrence rate were compared between the two groups. Follow-up data were obtained from hospital charts or by telephone.

Statistical analysis

Categorical parameters of each group were compared by χ^2 test, and continuous parameters were compared using independent-sample t test. All analyses were performed using SPSS 12.0. $P < 0.05$ was considered statistically significant.

RESULTS

Laparoscopic left hepatectomy was successfully performed in 28 patients. Of the 28 patients, 3 underwent laparoscopic left hemihepatectomy, cholecystectomy, common bile duct exploration and T tube drainage, 18 underwent left lateral segmentectomy, cholecystectomy and common bile duct exploration. Of the 18 patients, 6 underwent primary suture of common bile duct, 12 underwent T tube drainage, 5 underwent left lateral segmentectomy with cholecystectomy, 1 underwent left lateral segmentectomy, common bile duct exploration and T tube drainage, and 1 underwent left lateral segmentectomy. All the 28 patients in LLH group underwent intraoperative cholangioscopic bile duct exploration or stone removal. Of the 33 patients in OLH group, 5 underwent open left hemihepatectomy, cholecystectomy, common bile duct exploration and T tube drainage, 23 underwent left lateral segmentectomy, cholecystectomy and common bile duct exploration. Of the 23 patients, 4 underwent primary suture of common bile duct, 19 underwent T tube drainage. Twenty-eight patients in OLH group underwent cholangioscopic bile duct exploration with stones removed (Table 1). Intraoperative findings and postoperative pathology displayed hepatolithiasis, cholangiectasis of Couinaud, chronic inflammation and fibration in all patients.

The mean operation time was longer in LLH group than in OLH group (158 ± 43 min *vs* 132 ± 39 min, $P < 0.05$). The intraoperative blood loss in two groups was similar (180 ± 56 mL *vs* 184 ± 50 mL). One patient in OLH group was transfused with 2 units of concentrated red blood cells, no patient in LLH group received blood transfusion. Bile leakage occurred in 2 patients of LLH group, and healed automatically 3 and 5 d after operation. Pleural effusion, observed in 2 patients, disappeared after thoracentesis. In OLH group, seroperitoneum occurred in 1 patient, hepatic abscess in 1 patient and infection of incision in 3 patients. No significant difference was found in complication rate (14.2% *vs* 15.2%) and intermediate stone residual rate (17.9% *vs* 12.1%) between the two groups. The mean postoperative hospital stay time was shorter in LLH group than in OLH group (6.8 ± 2.8 d *vs* 10.2 ± 3.4 d, $P < 0.01$), and the serum transaminase was transiently increased and jaundice disappeared at discharge with no death occurred in both groups (Table 2).

Of the 16 patients in LLH group discharged with their T tubes, 11 underwent T extubation 28-35 d after operation, 5 underwent it 42-60 d after operation when residual stones were completely removed by choledochoscopy. Of the 27 patients in OLH group discharged with their T tubes, 23 underwent T extubation 28-40 d after operation, 4 underwent it 50-60 d after operation when residual stones were completely removed by choledochoscopy. Fifty-seven patients (93.4%) including 27 in LLH group and 30 in OLH group were followed up for 2-40 mo (mean 17 mo). Stone recurrence was found in 1 patient of LLH group and in 1 patient of OLH group. Intrahepatic biliary cancer occurred in 1 patient of OLH group 29 mo after operation, and was surgically removed in another hospital.

DISCUSSION

In 1991, Reich *et al*[10] used laparoscope to remove a benign tumor located at the edge of liver, raising the curtain on laparoscopic hepatectomy. In 1993, Wayand *et al*[11] performed laparoscopic segmentectomy (segments Ⅵ) for metastatic carcinoma. In 1996, Azagra *et al*[12] first performed laparoscopic left lateral lobectomy (segments Ⅱ and Ⅲ) for hepatic adenoma in 1 patient. With the refinement of laparoscopic instruments and accumulated

experience in laparoscopic hepatectomy, indications for laparoscopic hepatectomy have gradually expanded from small, peripheral and benign diseases to large, central and malignant diseases[13,14]. Lesions located in Couinaud including Ⅱ, Ⅲ, Ⅳa, Ⅴ and Ⅵ segments are the best indications for laparoscopic hepatectomy, and regular left lateral lobectomy is expected to become its gold standard[8,15]. Most intrahepatic bile duct stones, especially left intrahepatic bile duct stones, manifested as a regional distribution, are usually combined with liver fibrosis and atrophy, which is also a good indication for laparoscopic hepatectomy[8,16]. The inclusion criteria for LLH for hepatolithiasis in this study were (1) multiple stones in the left or left lateral intrahepatic ducts with fibrosis and atrophy of hepatic lobes or segments, possibly combined with extrahepatic bile duct stones or a few stones in the right intrahepatic ducts; and (2) except for lots of stones in the right intrahepatic ducts, severe acute cholangitis, hepatic abscess, stone incarceration in the lower part of common bile duct and bile duct neoplasms. We believe that only LLH rather than common bile duct exploration is required for simple left hepatolithiasis. Chen *et al*[16] has described that endoscopic retrograde cholangiopancreatography (ERCP) and endoscopic sphincterotomy (EST) for left hepatolithiasis with extrahepatic bile duct stones, can completely remove common bile duct stones followed by laparoscopic hepatectomy. However, ERCP easily causes acute pancreatitis, thus making cholelithiasis heavier and EST destroys the integrity of duodenal papilla and sphincter Oddi, easily leading to biliary tract infection and difficulty to remove the stones with a diameter > 1.5 cm. Preoperative hospital stay time is significantly increased and secondary operation is required. In this study, common bile duct exploration was performed to remove stones followed by T-tube drainage or primary suture of common bile duct, which is suitable for different sizes of common bile duct stones, but is time-consuming and may lead to bile leakage. External drainage of bile may lead to electrolytical and digestive unbalance, which is not beneficial to postoperative recovery. Choledochoscopy can remove the stones through the stump of the left hepatic duct without cutting open the common bile duct, thus avoiding the above disadvantages. However, it is only suitable for a small number of patients. In this study, a few stones in the right hepatic bile duct were removed by intraoperative choledochoscopy through the common bile duct or by postoperative choledochoscopy *via* the T-tube sinus tract.

The liver possesses dual blood supply from hepatic artery and portal vein. Blood supply is abundant and bleeding easily occurs. During laparoscopic hepatectomy, since hepatic portal occlusion, hand pressure and saturation cannot be used for hemostasis, it is not easy to control intraoperative bleeding[17]. Therefore, how to prevent, control and reduce intraoperative bleeding is a key to surgical success and postoperative recovery[18]. In our study, full preoperative preparation was done for each patient. The patients with normal liver function and coagulation test were selected. Preoperative ultrasonography, CT or MRCP was performed to determine the distribution, location and size of stones and liver morphology. The stones were located in Couinauds Ⅱ and Ⅲ. The operators had rich experience in traditional hepatectomy and skilled laparoscopic technique. Excellent surgical instruments such as ultrasonic bleeding knife, vascular closure device and Ligasure vessel sealing system were provided. Each segment of the left liver was dissected in the first hepatic portal, and blocked with clips. The portal vascular structure of left liver was dissected outside Glisson sheath, and the branches of portal vein and hepatic artery were clamped with absorbable clips. The left hepatic vein trunk was dissected, clamped with clips and divided after transection of liver parenchyma. The transaction of liver parenchyma resulted in less tissue damage, less bleeding and good exposure of hepatic duct system. It was reported that Peng's multifunctional operative dissector can reduce bleeding and operation time[19]. The ideal level of regular left lateral lobectomy is at the sagittal plane 1 cm away from the left of falciform ligament. The main vessels at this plane include superior and inferior segment branch of Couinaud of portal vein, trunk of left hepatic vein, left and upper branches of left hepatic vein, which are distributed in 2/3 of the superior part. Choledochoscope can enter the common bile duct or the right hepatic duct through the broken end of left hepatic duct or incisional anterior wall of the common bile duct to remove stones[6,7]. If no biliary tract stricture is identified, small stones may not be completely removed, and then a T tube is left. The residual small stones can be removed by choledochoscopy 6 wk after operation.

Hepatolithiasis is common in China, accounting for about 10% of calculosis, or 40%-50% in some areas. Stone clearance rate for open hepatectomy can reach 81.7% with a reliable long-term therapeutic effect[20]. Laparoscopic hepatectomy can achieve the same stone clearance rate in the left liver[6]. Zhang *et al*[8] reported that the therapeutic effect of laparoscopic left lateral lobotomy for left hepatolithiasis and choledocholith is better than that of traditional open stone removal. Machado *et al*[21] reported one patient who underwent laparoscopic right hepatectomy for hepatolithiasis, showing that the learning curve can influence the feasibility and repeatability of laparoscopic hepatectomy[22]. In this study, the therapeutic effects of laparoscopic and open hepatectomy on intrahepatic and extrahepatic bile duct stones were compared as previously described[9]. Although randomized controlled trial data are lack, the blood loss, blood transfusion rate, complication rate and mortality in laparoscopic hepatectomy were equal to those in open hepatectomy. It was reported that the evacuating time, fasting time, use of analgesics, hospital stay time, time to return to work and degree of satisfaction are better in laparoscopic hepatectomy than in open hepatectomy, while operation time is longer and cost is higher in laparoscopic hepatectomy than in open hepatectomy[5,23,24]. In our study, the mean operation time of LLH group was longer than that of OLH group, the mean blood

loss and postoperative complication rate were similar in the two groups, the mean hospital stay time was significantly shorter in LLH group (6.8 ± 2.8 d) than in OLH group (10.2 ± 3.4 d) possibly due to the mini-invasive advantages, such as small incision, postoperative light inflammatory response, less interference with immune function. In addition, intermediate and final stone clearance rate and long term stone recurrence rate for the two groups were also similar with no CO_2 gas embolism occurred in patients of LLH group. Air embolism is a potential risk factor for laparoscopic hepatectomy.

In summary, LLH for hepatolithiasis is safe and feasible in selected patients if it is performed by surgeons with experience in laparoscopic and hepatic surgery. Its therapeutic effect is equal to that of traditional open hepatectomy. Laparoscopic hepatectomy has the advantages such as mini-invasion, less postoperative pain and rapid recovery.

COMMENTS

Background

Hepatolithiasis is a prevalent disease in Southeast Asia with a high incidence in coast areas, southwest region, Hong Kong and Taiwan of China. Hepatectomy is a definite and effective procedure for hepatolithiasis. With the refinement of laparoscopic instruments and accumulated experience in laparoscopic and hepatic surgery, laparoscopic hepatectomy has been used in treatment of hepatic benign and malignant tumors and donor hepatectomy for live donor liver transplantation. However, few studies are available on on laparoscopic hepatectomy for hepatolithiasis.

Research frontiers

The feasibility and therapeutic effect of total laparoscopic left hepatectomy (LLH) on hepatolithiasis are a hotspot.

Innovations and breakthroughs

This study explored the therapeutic effect of laparoscopic *vs* open left hepatectomy on hepatolithiasis and concluded that LLH for hepatolithiasis is feasible and safe in selected patients if it is performed by experienced surgeons. Its therapeutic effect is equal to that of traditional open hepatectomy.

Applications

Laparoscopic hepatectomy possesses the advantages such as mini-invasion, less postoperative pain and rapid recovery and worth of spreading.

Terminology

Hepatolithiasis: It refers to stones in the branching bile ducts above the confluence of left and right hepatic ducts, and may occur alone or in combination with extrahepatic bile duct stones.

Peer review

It is interesting and well-written. The results show that laparoscopic hepatectomy is worth of spreading due to its advantages such as mini-invasion, less postoperative pain and rapid recovery.

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CASE REPORT

Eight-year survival after advanced gastric cancer treated with S-1 followed by surgery

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Abstract

We report a case of advanced gastric cancer, with cervical, axillary, and abdominal paraaortic lymph node metastases, that was successfully treated with chemotherapy and surgery. The disease was initially considered unresectable, and the patient was treated with orally administered S-1. Chemotherapy was effective, and all lymph node metastases disappeared after 6 courses. After 27 mo of chemotherapy, the patient underwent curative surgery, with subtotal gastrectomy and lymph node dissection. Histopathological examination revealed many viable poorly differentiated adenocarcinoma cells in the stomach, but no cancer cells in the lymph nodes. The patient is alive, without recurrence, 8 years later. This, therefore, is a case report of an 8-year survivor of advanced gastric cancer with distant lymph node metastasis.

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Key words: S-1; Lymph node metastasis; Gastric cancer; Gastrectomy

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Hijioka S, Chin K, Seto Y, Yamamoto N, Hatake K. Eight-year survival after advanced gastric cancer treated with S-1 followed by surgery. *World J Gastroenterol* 2010; 16(22): 2824-2827 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i22/2824.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i22.2824

INTRODUCTION

Improvements in early diagnosis of gastric cancer (GC) have led to an increase in the number of patients who are able to undergo curative resection[1]. However, the prognosis for patients with unresectable or metastatic disease is very poor, and long-term survival, particularly for more than 5 years, is rare. S-1 is a novel oral fluoropyrimidine that was developed in Japan. It has been available for patients with GC in Japan since 1999 and is currently being investigated worldwide. S-1 consists of tegafur, 5-chloro-2,4-dihydroxypyridine (CDHP) and potassium oxonate at a fixed molar ratio of 1:0.4:1. Tegafur is a prodrug of fluorouracil (5-FU), which is the cytotoxic component of this combination. CDHP is a potent reversible inhibitor of dihydropyrimidine dehydrogenase (DPD), the chief catabolic enzyme of 5-FU. Potassium oxonate selectively inhibits orotate phosphoribosyltransferase, the enzyme responsible for 5-FU activation in the gastrointestinal tract, thus reducing the gastrointestinal toxicity of the combination. Many reports have indicated the efficacy of this antitumor agent against gastric cancer.

We report a case of advanced gastric cancer (AGC) with unresectable lymph node metastases treated with S-1.

CASE REPORT

A 71-year-old man visited a local hospital in 2002, complaining of upper abdominal discomfort of one-month duration. Endoscopy revealed a concave lesion in the stomach, and the patient was subsequently referred to our hospital. Endoscopy showed a diffuse concave lesion with ulceration on the lesser curvature in the middle of the stomach. A biopsy specimen showed signet-ring cell carcinoma. Physical examination revealed a few elastic-hard masses in the left supraclavicular area (approximately 7 cm in diameter) and left axillary space (approximately 6 cm in diameter), suggesting lymph node metastases from the GC. Abdominal computed tomography (CT) showed marked swelling of several paraaortic lymph nodes (Figure 1A). We could not find metastasis to the liver, lung, or peritoneum. The patient's diagnosis was AGC with extensive lymph node metastases (stage Ⅳ: cT2 cN3 cP0 cH0 cM1). His general condition was good, with The Eastern Cooperative Oncology Group (ECOG) performance status 0, and biochemical analysis of blood and urine specimens showed no abnormalities. Serum levels of the tumor markers carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) were not elevated (2.1 ng/mL and 12.0 U/mL, respectively). Since curative resection was impossible for this patient, chemotherapy with S-1 was started. As one course, 120 mg (80 mg/m^2) of S-1 was orally administered daily for 28 d, followed by a 14-d rest period; administration occurred primarily at the outpatient clinic. After 2 courses, endoscopy showed no remarkable change in the gastric tumor. However, abdominal CT showed remarkable reductions in the size of regional and paraaortic lymph nodes (Figure 1B), and also in cervical and axillary lymph nodes (Figure 2A and B). This indicated a partial response (PR). The evaluation after 6 courses of S-1 showed that there was no change at the primary site, but CT showed complete disappearance of paraaortic, cervical and axillary lymph nodes. Therefore, this revealed a complete response (CR), and this condition was stable up to the subsequent surgery. The patient did not experience any critical adverse event during S-1 administration, despite developing hematological toxicity; including leucopenia (2100/μL at the minimum), thrombocytopenia, and non-hematological toxicity; including grade 2 hand-foot syndrome and grade 1 stomatitis, according to National Cancer Institute Common Toxicity Criteria Version 2.0. After 19 courses of S-1, the tumor was limited to the primary gastric site, with no metastasis detected from neck to abdomen by CT or positron emission tomography (PET); only the detection of gastric tumor by PET. Therefore, we concluded that curative resection could be achieved. In 2004, distal gastrectomy and lymph node dissection (D2) were performed. We did not find cancer cells in the intraoperative peritoneal lavage, or in the abdominal paraaortic lymph node specimens. Figure 3 shows the resected gastric specimen. A concave lesion of 45 mm × 40 mm with an ulceration scar was located in the lesser curvature of the middle of the stomach (Figure 3). Histopathologi-

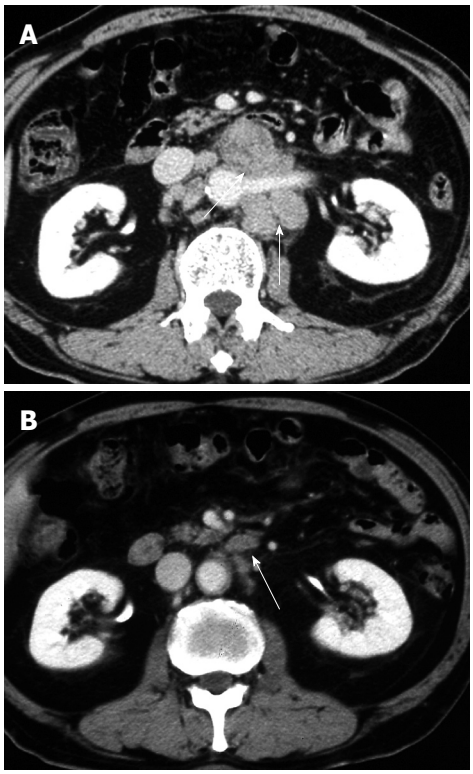


Figure 1 Abdominal computed tomography (CT). A: Marked swelling of paraaortic lymph nodes before treatment (arrow); B: After two courses of S-1, abdominal CT showed remarkable reductions (arrow).

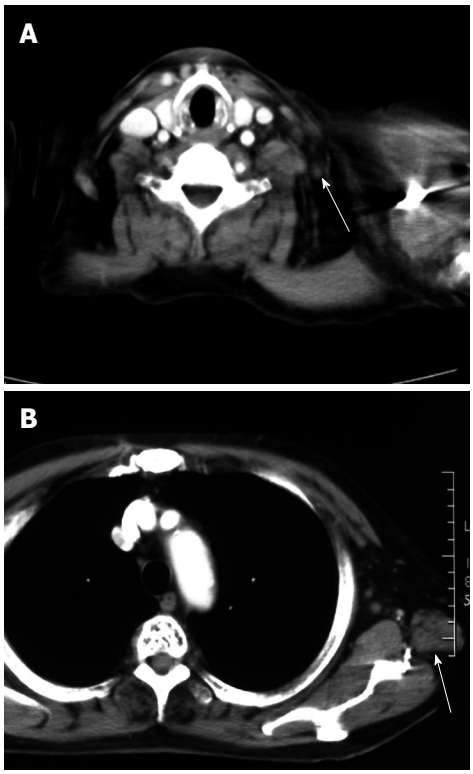


Figure 2 CT shows a Virchow's (A) and an axillary lymph node (B) which were reduced remarkably in size after treatment.

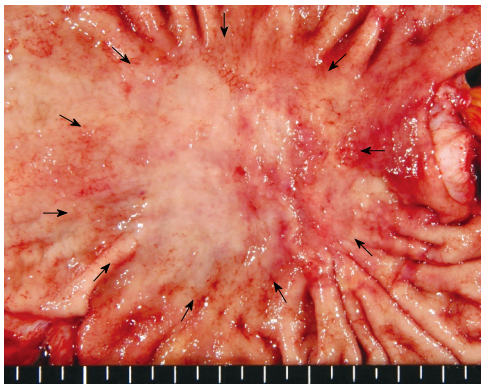


Figure 3 Resected specimen shows a concave lesion of 45 mm × 40 mm (arrows) with an ulceration scar which was located in the lesser curvature in the middle of the stomach.

cal examination showed numerous poorly differentiated adenocarcinoma cells in the mucosal layer (Figure 4A). There were only a small number of poorly differentiated adenocarcinoma cells, but severe fibrosis, in the submucosal layer (Figure 4B). The histological effect of chemotherapy on the primary tumor was limited, and according to the Japanese classification of gastric carcinoma (second English edition)[2], it was classified as grade 1a. The only findings in the lymph nodes were remarkable fibrosis and giant cells, with no cancer cells seen; the histological effect on the lymph nodes metastasis was classified as grade 3 (Figure 4C). Therefore, a radical operation was considered to have been performed, and the final pathological stage assigned as Stage IA (pT1, pN0, sP0, sH0, sM0) according to the Japanese classification of gastric carcinoma (second English edition)[2]. There have been no signs of recurrence since the surgery, and the patient has survived for 8 years after initially receiving chemotherapy.

DISCUSSION

There have been some series that reported high response rates with the use of newer combinations of chemotherapy regimens for unresectable or metastatic GC[3]. However, standard regimens have not yet been established worldwide. S-1-based chemotherapy has shown survival benefits in recent randomized controlled trials. Boku *et al*[4] reported that S-1 monotherapy showed a significant non-inferiority benefit compared to continuously infused 5-FU in unresectable or metastatic GC ($P < 0.001$). Moreover, Koizumi *et al*[5] reported the results of a randomized controlled trial of S-1 *vs* S-1 with CDDP. The overall survival for S-1 with CDDP was superior to S-1 alone. The 1- and 2-year survival rates were 54.1% and 23.6%, respectively; whereas a 2-year survival rate of less than 10% was shown in the 5-FU+CDDP arm of a Japanese randomized controlled trial with a 10-year follow-up[6].

Although recent phase Ⅲ clinical trials indicated longer survival times[7,8], few patients with unresectable GC have survived longer than 5 years. During the last 10 years at our hospital, only 9 of more than 500 patients treated with chemotherapy for metastatic or recurrent

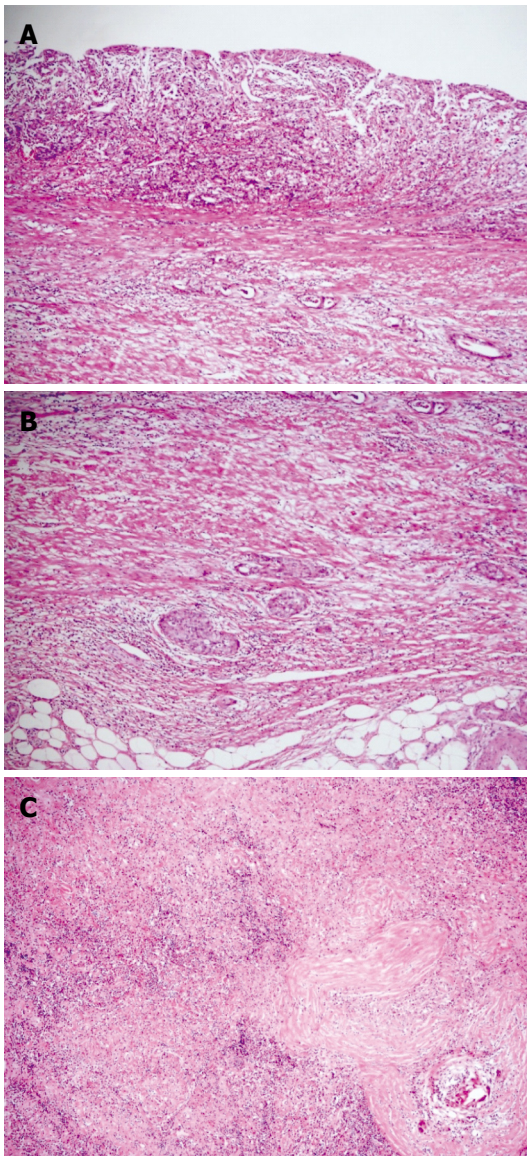


Figure 4 Histopathological examination. A: Histopathological examination showed numerous poorly differentiated adenocarcinoma cells in the mucosa; B: There were only a small number of poorly differentiated adenocarcinoma cells, but severe fibrosis, in the submucosal layer; C: The only findings in the lymph nodes were remarkable fibrosis and giant cells, with no cancer cells seen.

GC have survived for 5 years. Yoshida *et al*[9] reported a 5-year survival rate of 2% for registered Japan Clinical Oncology Group (JCOG) clinical trials and mentioned certain characteristics of these survivors. Most of the 5-year survivors had good performance status, macroscopically non-scirrhous-type tumors, only one metastatic site, a paraaortic node metastasis as the only unresectable factor, and achieved a CR after the initial chemotherapy. However, it is unclear whether such long survivals are because of biological non-aggressiveness or due to a good response to chemotherapy. There have been only five cases reported in the literature of AGC with neck lymph node metastasis in which there was no residual tumor following chemotherapy and surgical resection[10-14]. Ohyama *et al*[10] reported a case of a patient treated with combination chemotherapy, including 5-FU, leucovorin, cisplatin,

and etoposide, in which there was a good response. Chemotherapy was followed by curative resection, including subtotal gastrectomy and inguinal, neck, and abdominal lymph node dissection. Pathological examination showed cancer cells only in the abdominal lymph nodes, but not in the stomach or neck lymph nodes. Iwazawa *et al*[11] reported a case of AGC with cervical lymph node metastasis, in which a CR for cervical lymph nodes was achieved with S-1 alone treatment, and a curative resection of the residual tumor was performed. However, further outcomes of these cases were not described. It remains unknown whether adjuvant surgery for residual tumor after effective chemotherapy is beneficial for GC patients with distant metastasis or not. In our experience, if there is a long-maintained disappearance of inoperable metastasis, adjuvant surgery is considered beneficial.

In our case, there was no confirmation of the neck and axillary lymphadenopathy from imaging examinations before treatment began, and therefore no evidence that this lymphadenopathy was due to metastasis from GC. This is a limitation of our report and we assume responsibility for not obtaining all available evidence of metastasis from GC, although surgeons agreed on the non-operability of this patient in the preoperative conference. However, in patients with histologically diffuse type GC that is greater than 3 cm in size and which has invaded the submucosal layer, 7.5% have lymph node metastasis beyond the perigastric site[15]. Thus, our assumption that cervical and axillary lymphadenopathy was due to GC metastases was probably correct.

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CASE REPORT

Treating bilio-duodenal obstruction: Combining new endoscopic technique with 6 Fr stent introducer

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Key words: Biliary obstruction; Duodenal obstruction; Pancreatic cancer; Self-expandable metallic stent; Stent placement; Thin forward-viewing endoscope

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Abstract

Periampullary cancer may cause not only biliary but also duodenal obstructions. In patients with concomitant duodenal obstructions, endoscopic biliary stenting remains technically difficult and may often require percutaneous transhepatic biliary drainage. We describe a method of metal stent placement *via* a thin forward-viewing endoscope in patients with simultaneous biliary and duodenal obstruction. In two consecutive patients with biliary and duodenal obstruction due to pancreatic cancer, a new biliary metal stent mounted in a slim delivery catheter was placed *via* a thin forward viewing endoscope after passage across the duodenal stenosis without balloon dilation. In both patients, with our new placement technique, metallic stents were successfully placed in a short time without adverse events. After biliary stenting, one patient received curative resection and the other received duodenal stenting for palliation. Metallic stent placement with a forward-viewing thin endoscope is a beneficial technique, which can avoid percutaneous drainage in patients with bilio-duodenal obstructions due to periampullary cancer.

INTRODUCTION

Patients with periampullary malignancies occasionally experience not only biliary but also duodenal obstructions. Although biliary obstruction usually occurs first and is followed by duodenal obstruction, the two occur simultaneously in some cases. These patients often require percutaneous biliary stenting because the endoscopic procedure is difficult.

A new commercially available self-expandable metallic stent (SEMS) mounted in an extra slim delivery catheter can be passed through the working channel of a thin endoscope. Here, we assessed the outcome of metal stent placement *via* a thin forward-viewing endoscope in two patients with simultaneous biliary and duodenal obstruction.

CASE REPORT

Two consecutive patients with simultaneous biliary and duodenal obstructions due to pancreatic cancer between No-

Table 1 Summary of studied patients

Case	Age/sex	Level of duodenal obstruction	Level of biliary obstruction	Comorbidity	Purpose of biliary stenting	Subsequent procedures
1	79/F	Pars Ⅱ	Distal	None	Presurgical decompression	Pancreatico-duodenectomy
2	81/M	Pars Ⅱ	Distal	Advanced esophageal cancer	Palliation	Duodenal and esophageal stenting

vember 2009 and January 2010 were investigated (Table 1). In both patients, duodenal obstructions did not allow the passage of a duodenoscope across the stricture without a dilating procedure. The patients underwent placement of SEMS *via* a slim forward-viewing gastroscope (GIF XP-240, Olympus, Tokyo, Japan) (a valid length of 1030 mm, an outer diameter of 7.7 mm, a working channel diameter of 2.2 mm). The SEMS used is a new uncovered SEMS, Zilver® 635 stent (10 mm in diameter) (Cook, Bloomington, IN, USA) which is a laser-cut nitinol stent mounted in an extra slim (6-Fr) delivery catheter. The study was approved by our institutional review board (OHASHI #21-017), and written informed consent was obtained from the patients.

A slim endoscope was inserted perorally and passed across the stricture. The ampulla was endoscopically visualized by retroflexing the endoscope in the distal second portion of duodenum. An endoscopic retrograde cholangiopancreatography (ERCP) catheter (PR-109Q, Olympus) was advanced into the bile duct using a wire-guided cannulation technique with a 0.025 "hydrophilic guidewire (Surf®, Piolax Medical Devices, Inc. Kanagawa, Japan). Then, the guidewire was replaced with a 0.035" Jagwire (Boston Scientific Inc., Natick, MA, USA), endoscopic papillary balloon dilation was performed with an 8-mm balloon dilator (Eliminator® PET biliary balloon dilator, ConMed, Utica, NY, USA), and the SEMS was released under endoscopic and fluoroscopic control.

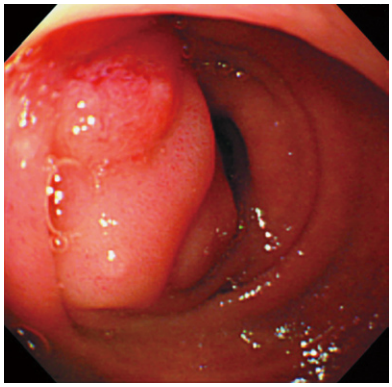


Figure 1 Endoscopic view of the forward-viewing endoscope showing duodenal stricture at the second portion of duodenum.

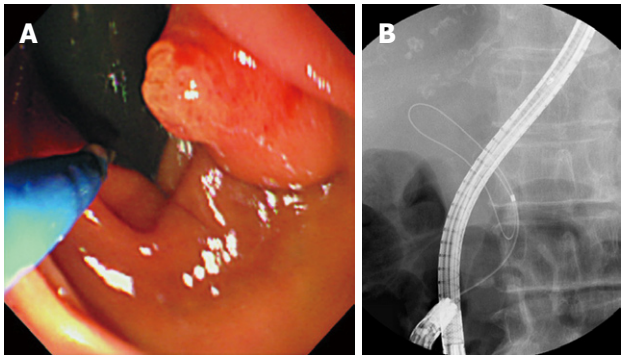


Figure 2 Selective biliary cannulation conducted with a thin forward-viewing endoscope. A: Endoscopic view showing endoscopic retrograde cholangiopancreatography (ERCP) catheter approaching to the papilla; B: X-ray picture showing common bile duct cannulation conducted with wire-guided technique without contrast injection.

Case 1

A 79-year-old woman was admitted to our hospital due to obstructive jaundice. Computed tomography (CT) revealed pancreatic cancer with duodenal invasion. The patient underwent ERCP for biliary stenting, during which a duodenoscope (JF-260V, 12.6 mm outer diameter, Olympus) could not be passed across the stricture in the second portion of duodenum. Despite the presence of duodenal stricture, she was able to take soft food orally.

The new stenting procedure with a thin forward-viewing endoscope was employed. In brief, a slim endoscope was inserted perorally and easily passed across the stricture (Figure 1). After successful biliary cannulation (Figure 2), a Zilver® 635 stent (8 cm in length) was placed and deployed at the optimal position (Figure 3). The entire procedure took 35 min, and no complications were encountered.

After biliary stenting, her cancer was considered resectable based on the assessment with detailed examinations. The patient successfully underwent pancreatico-duodenectomy on day 14 after stenting when her jaundice was relieved. The resected specimen revealed duodenal invasion from pancreatic cancer. Since then, the patient has received adjuvant chemotherapy with S-1.

Case 2

An 81-year-old man was admitted for epigastric pain, nausea and vomiting. Ultrasonography and CT revealed a 40-mm pancreatic mass with invasion to the extrahepatic bile duct, duodenum and portal vein. In addition, esophagogastroduodenoscopy revealed an advanced cancer in the lower part of esophagus and a duodenal stenosis due to pancreatic cancer. The cancer was therefore considered unresectable. Because of these gastrointestinal obstructions, the patient could not take food orally and both biliary and duodenal stenting for palliation were scheduled. Liver function tests showed abnormal results, although

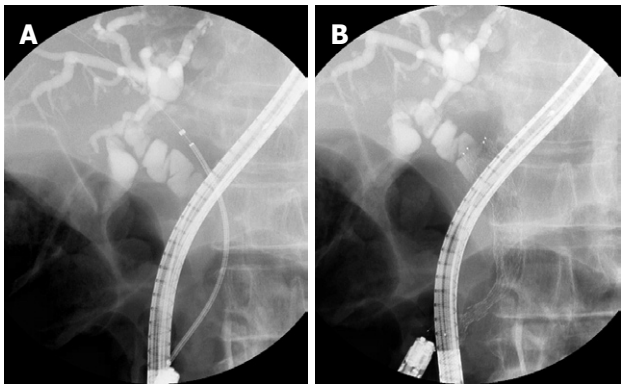


Figure 3 Biliary self-expandable metallic stent (SEMS) placement *via* a thin forward-viewing endoscope. A: An extra slim delivery catheter is inserted properly into the bile duct; B: SEMS is successfully placed.

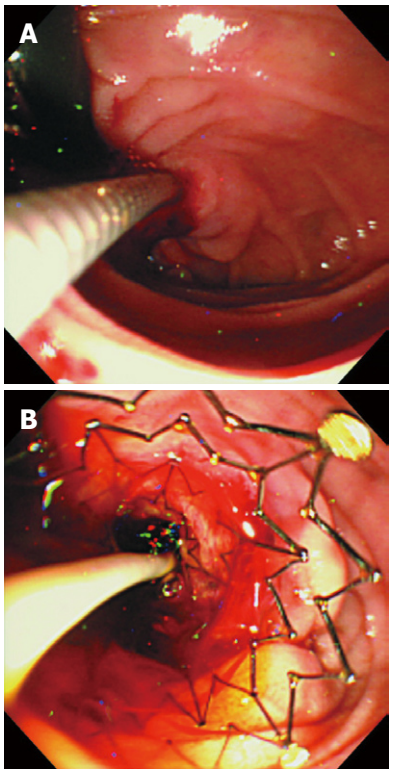


Figure 4 Endoscopic view showing biliary metallic stent placement *via* a thin forward-viewing endoscope. A: The delivery catheter is successfully introduced into the bile duct; B: SEMS is successfully placed.

jaundice was not identified. We carried out biliary stenting first in view of a treatment strategy based on scheduled palliative stenting, both biliary and duodenal[1].

Because it was impossible to pass a large-caliber side-viewing duodenoscope (JF-260V, Olympus) through the duodenal stricture, we employed the new biliary stenting procedure with a thin forward-viewing endoscope as in Case 1 and subsequently placed a duodenal stent. A thin endoscope was easily passed across the duodenal as well as esophageal obstruction, after which a Zilver® 635 stent (8 cm in length) was successfully placed without difficulty, even through the endoscope was retroflexed as in Case 1 (Figure 4). The total procedure time was 22 min.



Figure 5 X-ray picture indicating successful placement of biliary, duodenal and esophageal stents.

No procedure-related complications were found.

On day 10 after biliary stent placement, the patient underwent stent placement at the duodenal stricture. A duodenal stent (Niti-S® Pyloric/Duodenal Uncovered Stent, Taewoong Medical Inc. Seoul, Korea) was successfully placed with a through-the-scope placement procedure at the optimal position. Two weeks after the duodenal stenting, the patient also underwent stent placement at the esophageal obstruction with an Ultraflex™ (Boston Scientific) (Figure 5). Biliary, duodenal and esophageal stenting improved his dietary status, allowing intake of solid food.

DISCUSSION

We report here the successful endoscopic placement of a biliary SEMS using a thin forward-viewing endoscope in two patients with simultaneous biliary and duodenal obstructions. One patient underwent the procedure for presurgical decompression and the other for palliation.

The combination of biliary and duodenal obstruction in patients with periampullary cancer is relatively frequent, as 23% of the patients had both biliary and duodenal obstructions in a retrospective study of unresectable pancreatic head cancer[2]. In most cases, duodenal obstruction occurs after biliary obstruction, but simultaneous obstruction also occasionally occurs. Because stent placement in these simultaneous cases is more complicated, these patients often require percutaneous biliary stenting. Even if a duodenoscope can be passed across the duodenal stricture when a duodenal stent is first placed, access to the ampulla may be hampered by stent mesh covering the papilla. Biliary stenting before duodenal stenting is therefore recommended for these patients[1]. However, because the stricture is usually too narrow to allow passage of a large-caliber duodenoscope, hydrostatic balloon dilation is frequently required[2,3]. Even after dilation, passage of a side-viewing duodenoscope across the obstruction is sometimes difficult, and is at a risk for duodenal perforation owing to the presence of acute angulation in the duodenum. Because the endoscope used in the present study is much thinner and forward-viewing, we were able to pass it easily and safely across the stricture under direct vision. The present technique thus has the great advantage of allowing the easier and safer emplacement of a biliary stent than a previously published technique of biliary stenting after a duodenal dilation with a side-viewing duo-

denoscope in patients with combined biliary and duodenal obstruction[2,3].

Although usually selected when endoscopic stenting is difficult, percutaneous stenting has two drawbacks, namely a compromised quality of life owing to the need for an indwelling percutaneous transhepatic biliary drainage (PTBD) catheter for at least one week before stent placement, and the risk of cancer implantation in the catheter tract. A retrospective analysis of patients who underwent PTBD before resection of extrahepatic cholangiocarcinoma showed that catheter tract cancer-implantation develops in 6%[4]. Catheter tract cancer-implantation is reportedly observed in patients with pancreatic cancer as well as cholangiocarcinoma[5]. In recent years, endoscopic ultrasound (EUS)-guided biliary drainage has been introduced as an alternative to PTBD when ERCP is unsuccessful. However, this procedure is associated with procedure-specific complications, such as bile leak-attributable peritonitis[6,7], in addition to bleeding and cholangitis from stent dysfunction. Theoretically, it may cause cancer implantation in the peritoneal cavity and fistula track as in the case of PTBD. Transpapillary stenting is the only procedure conducted *via* a natural route and so must be the most desirable technique for presurgical as well as palliative decompression.

Recently, cholangiographic or cholangioscopic procedures with an ultra-slim endoscope have been reported[8,9]. A prospective comparison of transnasal ERCP with an ultrathin forward-viewing endoscope *vs* conventional ERCP with a large caliber side-viewing duodenoscope has reported no statistically significant difference in the rates or times of cannulation, albeit that the success rate of cannulation with the transnasal method is lower. The endoscope used in the present study (7.7 mm in diameter) is slightly larger than that used in the previous study (5.9 mm in diameter)[8]. Unlike the previous study, we introduced the endoscope into the duodenum perorally but were nevertheless able to accomplish biliary cannulation and stenting, without any difficulty. One previous case report of stent placement using an ultrathin forward-viewing endoscope *per os* has appeared[10], in which a patient with choledocholithiasis who received placement of two 5-Fr hand-made stents, subsequently underwent sphincterotomy and stone removal using a peroral ultrathin endoscope after relief of cholangitis. With plastic stents, thin endoscopes with a narrower working channel are restricted to accepting only 6 Fr or thinner stents. However, larger stents are generally more favorable than smaller ones, particularly for palliative use. We consider that the use of the new SEMS mounted in an extra slim delivery catheter takes full advantage of the possibilities of a thin endoscope with a thinner working channel.

When duodenal tumor invasion extends to the major papilla, endoscopic identification of the papilla is frequently difficult. Even if the papilla can be found, it is generally impossible to access the bile duct because of the difficulty of aligning the catheter with the bile duct axis. Under these conditions, therefore, stent placement would likely be difficult even with the present method and a percutaneous or EUS-guided transenteric procedure may be required instead. However, the number of such cases appears to be limited. The present report is a preliminary result with only two cases, and further study with more patients is warranted.

In conclusion, placement of the new SEMS mounted in an extra slim delivery catheter *via* a thin forward-viewing endoscope appears to be a safe and effective procedure for either presurgical or palliative decompression in patients with malignant biliary and duodenal obstruction. This study is preliminary, however, and further evaluation in a larger number of patients is warranted.

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CASE REPORT

Impaction of a lithotripsy basket during endoscopic lithotomy of a common bile duct stone

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Abstract

The treatments for common bile duct (CBD) stones are being continually developed. Impaction of the lithotripsy basket during endoscopic removal of CBD stones was seen in 5.9% patients. We report the case of a 66-year-old woman who underwent surgery for the removal of an impacted biliary basket. She was admitted to our hospital with a complaint of right upper abdominal pain. Magnetic resonance cholangiopancreatography revealed a CBD stone (20 mm × 15 mm). We diagnosed her with choledocholithiasis and performed endoscopic retrograde cholangiopancreatography to remove the stone. However, unfortunately, the retrievable basket around the stone became impacted. An endotriptor along with forceps could not be used owing to the entrapment of the basket, and thus we performed urgent surgery. The basket containing the stone was removed through a longitudinal choledochotomy. The wires leading to the basket were cut, and the basket containing the stone was removed *via* the incision. A T-tube was inserted, and the choledochotomy was closed. The postoperative course was uneventful. In conclusion, if the diameter of a CBD stone is more than 20 mm, then the risk of basket impaction increases, and surgery may be necessary as the initial treatment of the CBD stone.

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Key words: Common bile duct gallstones; Impacted biliary basket; Lithotripsy

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INTRODUCTION

Endoscopic procedures for the removal of common bile duct (CBD) stones are well established[1-3], and include endoscopic retrograde cholangiography (ERC), sphincterotomy, and basket or balloon extraction. However, endoscopic removal of large bile duct stones is difficult. The success or failure of lithotripsy depends on the size of the stone, number of stones, degree of jaundice, and presence or absence of gallbladder stone-induced cholecystitis. Here, we report the case of a patient with an impacted biliary basket.

CASE REPORT

A 66-year-old woman with right upper abdominal pain was admitted to our hospital. Laboratory analysis on admission

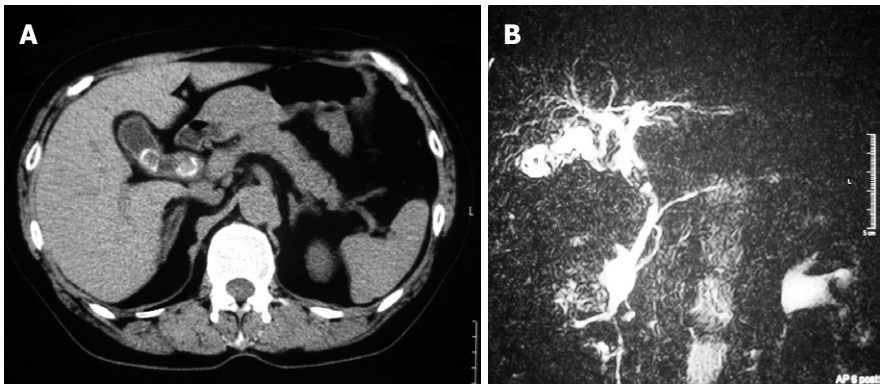


Figure 1 Computed tomography (CT) and magnetic resonance cholangiopancreatography (MRCP). A: Abdominal CT scan showing 2 stones in the gallbladder and a stone in the common bile duct; B: MRCP revealed a mass (20 mm × 15 mm in diameter) in the dilated common bile duct (20 mm in diameter).

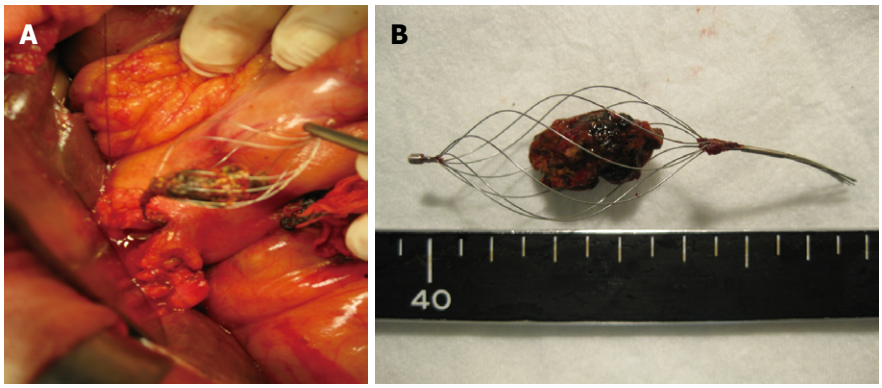


Figure 2 Intraoperative findings. A: During surgery, the impacted basket with stone were removed through a longitudinal choledochotomy; B: The size of the stone in the basket was 20 mm in diameter.

revealed elevated serum levels of the following biological parameters (the normal range for each parameter is in parentheses): alkaline phosphatase, 486 IU (109-335 IU); γ-glutamyl transpeptidase, 373 IU (12-48 IU); L-aspartate aminotransferase, 913 IU (12-32 IU); L-alanine aminotransferase, 449 IU (10-40 IU); total bilirubin, 2.3 mg/dL (0.2-1.4 mg/dL); and direct bilirubin, 1.3 mg/dL (0.0-0.3 mg/dL). The results of a complete blood count test and coagulation test were normal. The serum levels of tumor markers (carbohydrate antigen 19-9 and carcinoembryonic antigen) were within the normal ranges. Ultrasound examination and computed tomography revealed 2 stones in the gallbladder along with thickening of the gallbladder and a calcified stone in the CBD (Figure 1A). In addition, magnetic resonance cholangiopancreatography revealed a mass (20 mm × 15 mm in diameter) in the dilated CBD, which had a diameter of 20 mm (Figure 1B). We diagnosed the patient with choledocholithiasis-induced cholangitis and choledocholithiasis-induced cholecystitis. We planned endoscopic lithotripsy for the removal of the CBD stone as the initial treatment, followed by laparoscopic cholecystectomy.

ERC revealed the CBD stone as a large defect filling the upper part of the CBD. We performed endoscopic stone extraction, because the biliary tract was dilated after the endoscopic lithotripsy. The metal spiral sheath advanced to the basket containing the entrapped stone. However, unfortunately, the retrievable basket around the stone became impacted in the middle of the CBD. It was impossible to crush the stone because it had hardened owing to calcification; in addition, it was impossible to shut the opening wire and to replace the basket catheter with an endotriptor.

Subsequently, because the basket was trapped, we performed urgent surgery involving cholecystectomy and choledochotomy, because, in our department, we did not have an extracorporeal shock-wave lithotripsy.

During the operation, cholecystectomy was performed, and the CBD was explored. The basket containing the stones was removed through a longitudinal choledochotomy (Figure 2). The wires leading to the basket were cut, and the basket containing the stone was removed *via* the incision. Intraoperative choledochoscopy revealed no residual stones or fragments. A T-tube was inserted, and the choledochotomy was closed. The postoperative course was uneventful and the patient is clinically healthy.

DISCUSSION

The treatment of CBD stones has advanced from choledochotomy to endoscopic management, with a success rate of over 90% for the latter[4,5]. The techniques used for the management of CBD stones are endoscopic sphincterotomy and endoscopic papillary balloon dilatation. However, the complications of endoscopic management are hemorrhage, pancreatitis, sepsis, cholangitis, and occasionally, impaction of the lithotriptor basket during the endoscopic removal of a CBD stone. The reported incidence of impaction of a basket with an entrapped stone was 5.9%[4,6,7]; however, because of the developments in the therapeutic techniques for CBD stones, this incidence has decreased to 0.8%[8].

The main factor responsible for impaction of the

basket was reported to be the large size of the stone[4,6,7]. In the treatment of CBD stones with a diameter over 10-12 mm, a crush technique using endoscopic mechanical lithotripsy (EML) is employed, and the crushed stone is removed[9].

EML has been successfully used in 80%-90% of cases to crush CBD stones that were too large to be removed using conventional methods[10,11]. However, the management of very large stones with diameters of more than 25 mm, and of multiple stones in the ductus choledochus has failed[5]. In 4 studies that reported impaction of the basket, the average size of the stone was 17 mm (range, 13-20 mm)[4,7,12,13]. The success rate of EML for the treatment of large stones with diameters over 20 mm, was reported to be approximately 50%. However, the success rate is low if there are multiple stones and/or calcified stones. In our patient, the diameter of the stone was 20 mm: in addition, the stone was calcified and entrapped, and therefore could not be crushed and released in the lower part of the CBD. Impaction of the basket or CBD stone causes obstructive jaundice; furthermore, the likelihood of acute obstructive suppurative cholangitis, acute pancreatitis, and sepsis is great[14]. In the worst cases, failure of stone removal may result in the death of the patient. Garg *et al*[15] reported that stone size alone may not be an important factor that decides the success of EML; CBD dilatation with adequate working space between the stone and the wall of the CBD is also an important factor. In our patient, the size of the CBD stone was 20 mm and the CBD was dilated 20 mm, thus, there was no adequate space between the stone and the CBD wall to open the wire basket fully, and this may be one of the causes of failure of EML. In addition, cholecystitis with gallbladder stones may induce inflammatory changes in the CBD, leading to stiffness of the CBD, and, as a result, no adequate working space between the stone and the wall of the CBD.

In conclusion, we determined that if the size of CBD stone is over 20 mm in diameter and is calcified, the risk of an impacted basket is increased. In addition, if the CBD is not adequately dilated, there is insufficient space between the stone and the CBD wall to open the wire basket fully. In these cases, surgery may be selected as an initial treatment of CBD stones to prevent impaction of a basket.

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Meetings

Events Calendar 2010

January 25-26
Tamilnadu, India
International Conference on Medical Negligence and Litigation in Medical Practice

January 25-29
Waikoloa, HI, United States
Selected Topics in Internal Medicine

January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology Conference

January 28-30
Hong Kong, China
The 1st International Congress on Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of Gastroenterology & Endoscopy Meeting

March 09-12
Brussels, Belgium
30th International Symposium on Intensive Care and Emergency Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian National Association for Study of the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on Diabetes PACD14

March 25-28
Beijing, China
The 20th Conference of the Asian Pacific Association for the Study of the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology and Hepatology Conference, EGHC 2010

April 14-17
Landover, Maryland, United States
12th World Congress of Endoscopic Surgery

April 14-18
Vienna, Austria
The International Liver Congress™ 2010

April 28-May 01
Dubrovnik, Croatia
3rd Central European Congress of surgery and the 5th Croatian Congress of Surgery

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual Meeting

May 06-08
Munich, Germany
The Power of Programming: International Conference on Developmental Origins of Health and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and Rectal Surgeons Annual Meeting

June 04-06
Chicago, IL, United States
American Society of Clinical Oncologists Annual Meeting

June 09-12
Singapore, Singapore
13th International Conference on Emergency Medicine

June 14
Kosice, Slovakia
Gastro-intestinal Models in the Research of Probiotics and Prebiotics-Scientific Symposium

June 16-19
Hong Kong, China
ILTS: International Liver Transplantation Society ILTS Annual International Congress

June 20-23
Mannheim, Germany
16th World Congress for Bronchoesophagology-WCBE

June 25-29
Orlando, FL, United States
70th ADA Diabetes Scientific Sessions

August 28-31
Boston, Massachusetts, United States
10th OESO World Congress on Diseases of the Oesophagus 2010

September 10-12
Montreal, Canada
International Liver Association's Fourth Annual Conference

September 11-12
La Jolla, CA, United States
New Advances in Inflammatory Bowel Disease

September 12-15
Boston, MA, United States
ICAAC: Interscience Conference on Antimicrobial Agents and Chemotherapy Annual Meeting

September 16-18
Prague, Czech Republic
Prague Hepatology Meeting 2010

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Prague, Czech Republic
The 1st World Congress on Controversies in Gastroenterology & Liver Diseases

October 07-09
Belgrade, Serbia
The 7th Biannual International Symposium of Society of Coloproctology

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ACG 2010: American College of Gastroenterology Annual Scienitfic Meeting

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18th United European Gastroenterology Week

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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

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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Issue with no volume

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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)

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/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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EDITORIAL

Plexiform angiomyxoid myofibroblastic tumor of the stomach

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Abstract

Plexiform angiomyxoid myofibroblastic tumor of the stomach is a unique mesenchymal tumor that we first described in 2007. The tumor is very rare, and to date, only 18 cases confirmed by immunohistochemistry have been reported in the literature. The patients' ages ranged from 7 to 75 years (mean, 43 years), and the male-to-female ratio was approximately 1:1. Representative clinical symptoms are ulceration, associated upper gastrointestinal bleeding (hematemesis), and anemia. The tumors are located at the antrum in all cases, and grossly, the tumor is whitish to brownish or reddish, and forms a lobulated submucosal or transmural mass. Microscopically, the tumor is characterized by a plexiform growth pattern, the proliferation of cytologically bland spindle cells, and a myxoid stroma that is rich in small vessels and positive for Alcian blue stain. Immunohistochemically, the tumor cells are positive for α-smooth muscle actin and negative for KIT and CD34. Differential diagnoses include gastrointestinal stromal tumor and other mesenchymal tumors of the gastrointestinal tract. Some authors proposed that this tumor should be designated as "plexiform fibromyxoma", but this designation might cause confusion. The tumor is probably benign and thus far, neither recurrence nor metastasis has been reported.

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Key words: Plexiform angiomyxoid myofibroblastic tumor; Stomach; Gastrointestinal stromal tumor; Plexiform fibromyxoma; Myofibroblast; Fibroblast

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INTRODUCTION

The majority of gastrointestinal mesenchymal tumors are gastrointestinal stromal tumors (GISTs) that are characterized by a high frequency of positive immunohistochemical reactivity for KIT and mutations of the *KIT* gene[1-6]. Mutations of the platelet-derived growth factor receptor α (*PDGFRA*) gene have been reported in GISTs that are negative for mutations in *KIT*[7,8]. The effect of molecular target therapy by imatinib mesylate was reported for GIST[9,10], and in recent times, the accurate pathological diagnosis of GIST has been gaining more importance.

Mesenchymal tumors other than GIST occur in the stomach although their frequency is very low. In 2007, we reported 2 cases of a unique gastric mesenchymal tumor that had not been previously described and designated it as "plexiform angiomyxoid myofibroblastic tumor (PAMT)" of the stomach. The tumor was characterized by a plexiform growth pattern, the proliferation

of bland spindle cells that were separated by an abundant intercellular myxoid matrix, and a myxoid stroma that was rich in small vessels and histochemically positive for Alcian blue stain[11]. Since then, several similar cases have been reported[12-14]. Some authors reported cases with more obvious smooth muscle differentiation of the tumor cells, and used the term "plexiform angiomyxoid tumor" removing the "myofibroblastic" designation[14]. Furthermore, Miettinen *et al*[15] recently reported 12 cases of the same tumor under the diagnostic name of "plexiform fibromyxoma".

In this article, we review the clinicopathological features of PAMT cases, including an unpublished case that we recently examined, and discuss differential diagnoses, the nature of the tumor cells, and the appropriate nomenclature.

CLINICAL FEATURES

Epidemiology

PAMT of the stomach is a very rare tumor. To date, only 18 cases of PAMT of the stomach confirmed by immunohistochemistry have been reported in the medical literature[11-15]. Miettinen *et al*[15] estimated that the frequency of PAMT is less than 1/150 compared with that of gastric GIST. Table 1 shows the clinicopathological features of the reported cases and our new unpublished case of PAMT. The patients' ages ranged from 7 to 75 years (mean, 43 years). Ten cases were male and 9 cases were female; thus, the male-to-female ratio is approximately 1:1.

Symptoms and signs

The representative clinical symptoms are ulceration, associated upper gastrointestinal bleeding (hematemesis), and anemia. Other clinical symptoms include nausea, emesis, weight loss, gastric mass, and pyloric obstruction. One case was found by acute abdominal pain due to perforation[11], and one case was incidentally found during a cholecystectomy[11].

Endoscopic and radiographic findings

The tumor is detected as a submucosal tumor-like elevated lesion by endoscopy and is often ulcerated. The tumor was also detected as a multinodular hypoechogenic lesion[13] or as an infiltrating mass[14] using ultrasonography. In one case with a very large tumor, it was detected as a solid and cystic tumor using abdominal computed tomography (unpublished case).

PATHOLOGICAL FEATURES

Gross appearance

The size of PAMT of the stomach ranges from 1.9 cm to 15 cm, with a mean value of 6.3 cm. Interestingly, the tumor was located in the antrum in all 19 cases. The tumor often involves the pylorus, and extends to the duodenal bulb in approximately one-third of cases. The tumor is whitish to brownish or reddish, and forms a lobulated submucosal or transmural mass. It is not encapsulated and protrudes from the serosa (Figure 1A) in many cases, which often has a granular or nodular appearance. The surface of the tumor is ulcerated in approximately two-thirds of cases, and is perforated in one case (Figure 1B)[11]. Cystic degeneration was observed in the case with a very large tumor mass (unpublished case).

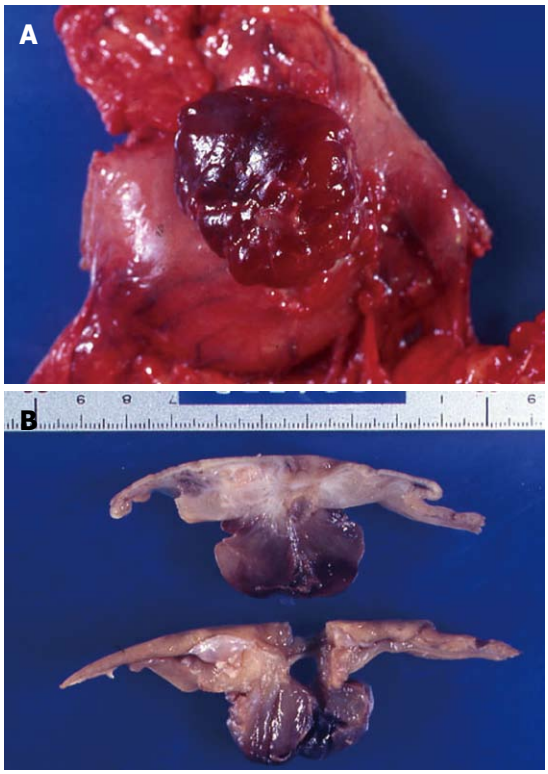


Figure 1 Gross appearance of a case of plexiform angiomyxoid myofibroblastic tumor (PAMT). A: A reddish and lobulated tumor protrudes from the serosa; B: The cut section of the tumor. The tumor causes ulceration and perforation.

Microscopic findings

PAMT shows a characteristic multinodular plexiform growth pattern in the gastric wall (Figure 2A). However, the extragastric tumor component does not show a plexiform pattern. The mucosa is often ulcerated. Spindle-shaped bland tumor cells are separated by an abundant intercellular myxoid or fibromyxoid matrix (Figure 2B). Fascicular or palisading arrangements of tumor cells are not usually observed. The tumor cells possess oval nuclei and a slightly eosinophilic cytoplasm. The nucleoli are inconspicuous and the cell borders are indistinct (Figure 2C). In the cases reported by Yoshida *et al*[14], a small number of tumor cells had morphological features reminiscent of smooth muscle cells (blunt nuclear ends and thin long eosinophilic cytoplasmic extensions). Mitoses are usually not detected (at most 4/50 high-power fields). The stroma is positive for Alcian blue stain (Figure 2D), and rich in small and thin-walled blood vessels. Dilated and arborizing blood vessels were also observed, and thrombi were formed in one case (unpublished case). Stromal collagenization was inconspicuous in some cases, but conspicuous in others (Figure 2E). The tumor cells

Table 1 Clinicopathological data of plexiform angiomyxoid myofibroblastic tumor of the stomach

Case	Age (yr)	Sex	Clinical presentation	Location	Tumor size (cm)	Mitoses	Ki-67 index	Operation	Prognosis	Ref.
1	50	M	Acute abdominal pain (due to perforation)	Antrum	4.0 × 4.0 × 2.5	None	< 1%	Distal gastrectomy	No data	[11]
2	68	M	None (incidental finding at cholecystectomy)	Antrum	4.5 × 3.5 × 3.0	None	< 1%	Partial gastrectomy	Alive without tumor (12 mo)	[11]
3	50	F	Morning nausea	Antrum	1.9 × 1.8 × 0.8	None	2%	Local excision (wedge resection)	Alive without tumor (3 mo)	[12]
4	61	M	Hematemesis	Antrum	3.7	None	Not stated	Partial gastrectomy	Alive without tumor (3 mo)	[13]
5	19	F	Mass in the stomach	Antrum	4.5 × 3.5 × 3.0	None	Not stated	Distal gastrectomy	Alive without tumor (9 mo)	[14]
6	46	M	Upper gastrointestinal bleeding	Antrum	3.5	< 1/50 HPF	< 1%	Distal gastrectomy	Alive without tumor (4 mo)	[14]
7	38	F	Upper gastrointestinal bleeding, ulcer	Antrum	3 × 2	1/50 HPF	Not stated	Distal gastrectomy	No data	[15]
8	62	M	Weight loss	Antrum	4 × 4	0/50 HPF	Not stated	Partial gastrectomy, omentectomy	No data	[15]
9	75	F	Unknown	Antrum	5	0/50 HPF	Not stated	Subtotal gastrectomy	Died of unknown cause (2 mo)	[15]
10	65	F	Weight loss, gastric ulcer	Antrum, duodenal bulb	5.0 × 4.5 × 2.5	4/50 HPF	Not stated	Partial gastrectomy	Died of unknown cause (14.5 yr)	[15]
11	33	M	Anemia, weakness	Antrum	5.5 × 3.5 × 3.5	0/50 HPF	Not stated	Distal gastrectomy	Alive without tumor (19.7 yr)	[15]
12	43	M	Gastrointestinal bleeding	Antrum	5.5 × 4.5 × 4.5	0/50 HPF	Not stated	Partial gastrectomy	Alive without tumor (18.4 yr)	[15]
13	56	F	Unknown	Antrum, duodenal bulb	5.5 × 3.0	0/50 HPF	Not stated	Partial gastrectomy	Alive without tumor (19.9 yr)	[15]
14	50	M	Gastric outlet obstruction	Antrum, duodenal bulb	7 × 6 × 6	2/50 HPF	Not stated	Distal gastrectomy	Died of unknown cause (25.5 yr)	[15]
15	21	M	Syncope, anemia	Antrum, duodenal bulb	9 × 6 × 5	0/50 HPF	Not stated	Antrectomy	Alive disease status unknown (22 yr)	[15]
16	16	F	Hematemesis	Antrum	10 × 9 × 6	2/50 HPF	Not stated	Distal gastrectomy	Alive disease status unknown (3 yr)	[15]
17	30	F	Gastric ulcer	Antrum	10 × 9 × 6	1/50 HPF	Not stated	Distal gastrectomy	Alive disease status unknown (24 yr)	[15]
18	7	F	Emesis, diarrhea, abdominal mass	Antrum, duodenal bulb	15 × 11 × 8	4/50 HPF	Not stated	Excision of tumor	No data	[15]
19	23	M	Abdominal pain and discomfort, tarry stool	Antrum, duodenal bulb	14 × 14 × 7	None	< 1%	Partial gastrectomy	Alive without tumor (12 mo)	Unpublished

HPF: High power fields.

are sometimes surrounded by a pseudovacuolar lacunar space limited by a fibrous matrix network. Mast cells are scattered in the stroma, but infiltration of lymphocytes, plasma cells, or eosinophils is inconspicuous. Tumor necrosis is not usually observed, but hemorrhage, necrosis, and cystic degeneration were observed in one case (unpublished case).

Immunohistochemical staining

In all cases examined, the tumor cells were positive for vimentin and muscle actin, and negative for KIT (Figure 3A), CD34, S-100 protein, neurofilament, cytokeratin, epithelial membrane antigen, and ALK. Tumor cells were positive for α-smooth muscle actin (SMA) in 15 of the 17 cases examined (Figure 3B), but a significant SMA-negative component was found in 3 cases. Focal or partial immunoreactivity for desmin and caldesmon was occasionally observed. Tumor cells were focally positive for CD10 in 1 of the 4 cases examined. The Ki-67 labeling index is usually less than 1% (at most 2%).

Mutation analysis of the KIT and PDGFRA genes

Sequence analysis was performed for mutational hot spots in the *KIT* and *PDGFRA* genes in GISTs (i.e. exons 9, 11, 13, and 17 of *KIT* and exons 12 and 18 of *PDGFRA*) in 8 cases, and no mutations were found in any case[11,12,14,15].

Differential diagnosis

The primary histological differential diagnoses include GIST, leiomyoma, leiomyosarcoma, schwannoma, desmoid fibromatosis, solitary fibrous tumor (SFT), inflammatory fibroid polyp, and inflammatory myofibroblastic tumor.

GISTs consist of spindle and/or epithelioid cells. In tumor areas composed of spindle cells, these cells are arranged in short fascicles in several architectural patterns including storiform, herringbone palisades, and broad sheets. Conversely, epithelioid cells are arranged in organoid clusters or sheets. Approximately 90% of GISTs are immunohistochemically positive for KIT, and muta-

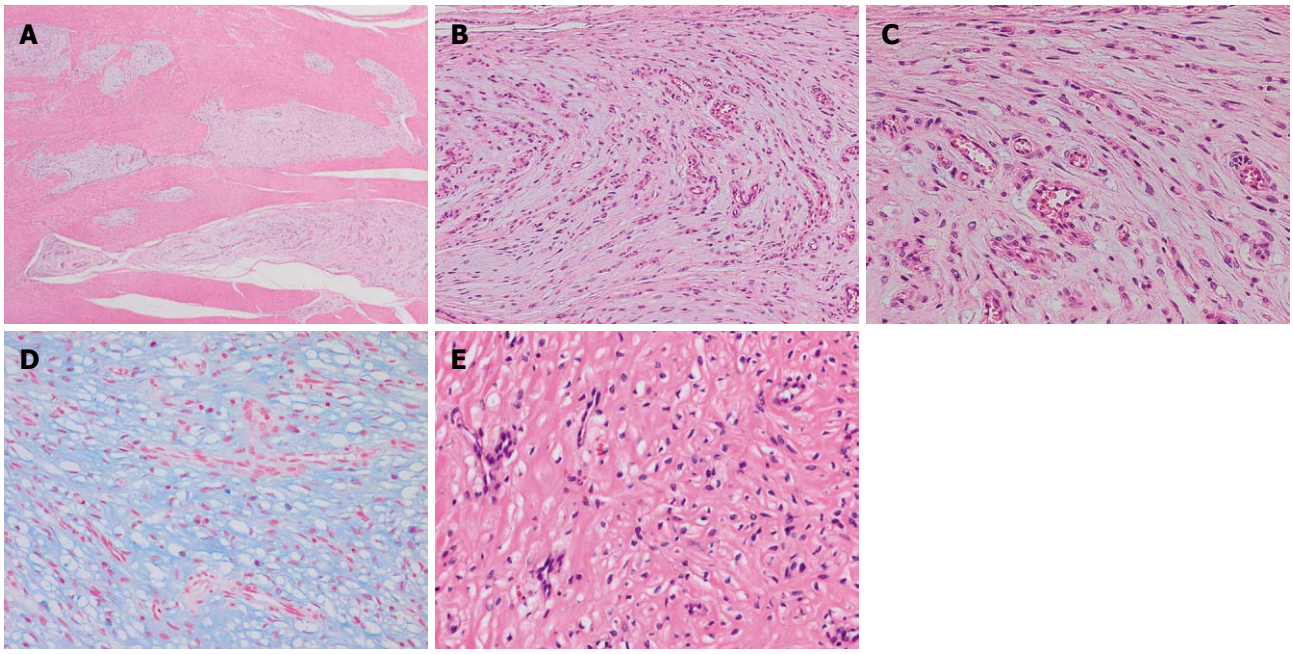


Figure 2 Histological appearance of PAMT. A: The tumor shows a multinodular plexiform growth pattern (HE stain, × 20); B: Spindle-shaped bland tumor cells are separated by an abundant intercellular myxoid matrix. The stroma is rich in small vessels (HE stain, × 100); C: Tumor cells possess oval nuclei and a slightly eosinophilic cytoplasm. The nucleoli are inconspicuous and the cell borders are indistinct (HE stain, × 200); D: The stroma is positive for Alcian blue stain (× 200); E: Stromal collagenization is occasionally observed (HE stain, × 200).

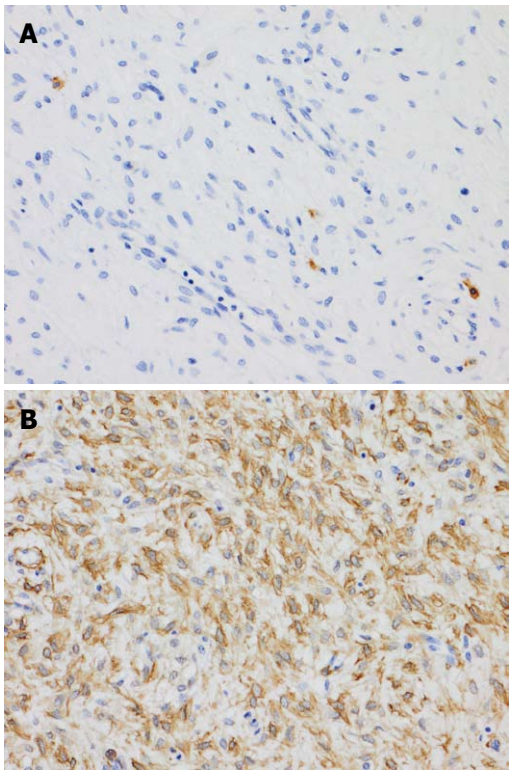


Figure 3 The results of immunohistochemical staining. A: The tumor cells are negative for KIT. The scattered KIT-positive cells are mast cells (× 200); B: The tumor cells are diffusely positive for smooth muscle actin (× 200).

tions of the *KIT* gene have been found in most of these KIT-positive GISTs[1-6]. *PDGFRA* gene mutations have been reported in some GISTs in the absence of *KIT* gene mutations[7,8]. In addition, immunohistochemical staining for CD34 is often positive in GISTs[16]. Accordingly, the histological appearance, negative immunoreactivity for KIT and CD34, and wild-type sequences of the *KIT* and *PDGFRA* genes differentiate PAMT from GIST.

Leiomyoma is characterized by the fascicular arrangement of tumor cells that possess spindle-shaped nuclei and markedly eosinophilic cytoplasm. Leiomyosarcoma shows high cellularity, cellular atypia, and remarkable mitoses. Gastric schwannoma is histologically characterized by wavy and palisading arrangements of spindle cells, lymphoid cuffing, and S-100 protein immunostaining. Desmoid fibromatosis shows long fascicular arrangements of spindle cells and dense collagen deposits. The alteration of hypercellular and hypocellular areas, deposition of dense keloid-type collagen, occurrence of hemangiopericytoma-like areas, and positive immunohistochemical staining for CD34 are the most distinguishing features of SFT. An inflammatory fibroid polyp is usually a small submucosal lesion characterized by bland spindle-cell proliferation in an onion skin-like pattern around vessels with eosinophilic infiltrates. Prominent lymphocyte and plasma cell infiltration, and positive immunoreactivity for ALK are the distinguishing features of inflammatory myofibroblastic tumors.

CELL NATURE AND NOMENCLATURE

In general, myofibroblasts are immunohistochemically positive for SMA, and negative for desmin and caldesmon[17]. Conversely, fibroblasts are negative and smooth muscle cells are positive for these three markers. Accordingly, the abovementioned immunohistochemical

characteristics of PAMT suggest that myofibroblastic tumor cells are predominant in most cases. The myofibroblastic nature of the tumor cells was also confirmed by ultrastructural analysis of PAMT[11]. However, the facts that focal immunoreactivity for desmin and caldesmon is occasionally observed in PAMT and the presence of a small number of tumor cells showing morphological features reminiscent of smooth muscle cells observed in some PAMT cases[14] suggest that PAMT may contain tumor cells with smooth muscle differentiation. Furthermore, Miettinen *et al*[15] confirmed the presence of tumor cells with fibroblastic traits. Accordingly, it is conceivable that PAMT is mainly composed of tumor cells with a myofibroblastic nature, but may also contain tumor cells with fibroblastic or smooth muscle characteristics.

We designated this tumor as "plexiform angiomyxoid myofibroblastic tumor" based on the plexiform growth pattern, a myxoid stroma that is rich in small vessels, and the myofibroblastic nature of the tumor cells[11]. Yoshida *et al*[14] reported cases with focal smooth muscle differentiation and used the term "plexiform angiomyxoid tumor", because the "myofibroblastic" designation might not be totally accurate. As mentioned above, we agree that this tumor is not a pure myofibroblastic tumor and may contain tumor cells with a fibroblastic or smooth muscle nature. However, we think that the "myofibroblastic" designation is not necessarily inappropriate, because myofibroblastic tumor cells are predominant in the majority of the reported cases. For example, inflammatory myofibroblastic tumors may also contain fibroblastic components as determined using ultrastructural analysis[18,19], and focally express desmin and calponin in 60%-70% of cases[18,20]. Miettinen *et al*[15] proposed that this tumor should be designated as "plexiform fibromyxoma" based on the plexiform architecture and combination of myxoid and fibromyxoid elements. However, most of the cases reported under the designation of "gastric fibromyxoma" or "gastric myxofibroma" were reported before the development of immunohistochemical techniques, and most of these cases are probably examples of other types of mesenchymal neoplasms with secondary regressive changes[21]. Currently, the term "gastric fibromyxoma" or "gastric myxofibroma" is used only in the case of pure fibroblastic tumors[22,23], and some different features from PAMT, such as positive immunoreactivity for CD34, are observed[22]. Superficial acral fibromyxomas possess different immunohistochemical features from those of PAMT, such as positive staining for CD34 and negative staining for SMA[24]. Thus, in using the designation "plexiform fibromyxoma", it is necessary to be careful to avoid confusion.

BIOLOGICAL BEHAVIOR AND PROGNOSIS

Follow-up data were obtained for 15 cases. The follow-up period ranged from 2 mo to 25.5 years (median, 3 years), and 12 patients were alive while 3 had expired. These 3 patients died 2 mo, 14.5 years, and 25.5 years after surgery, and the cause of death was unknown. Nine patients were alive without disease after 3 mo to 19.9 years (median, 12 mo), and 3 were alive for 3, 22, and 24 years with an unknown tumor status. Thus, neither recurrence nor metastasis was confirmed in any case.

CONCLUSION

PAMT of the stomach is a very rare mesenchymal tumor with a unique histological appearance, and it needs to be distinguished from GIST and other gastrointestinal mesenchymal tumors. It is very interesting that all of the reported PAMT cases were located at the gastric antrum, implying that this tumor might originate from a cell that is specifically distributed at this location. The absence of atypia of the tumor cells and a very low Ki-67 labeling index suggest the benign nature of this tumor, and neither recurrence nor metastasis was observed in any case. The clinicopathological features of the tumor should be elucidated by examination of a larger number of cases, and analysis of pathogenesis should be performed in the future.

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TOPIC HIGHLIGHT

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Endoscopic ultrasound for the diagnosis of chronic pancreatitis

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Abstract

Endoscopic ultrasound (EUS) has become a well accepted test for the diagnosis of chronic pancreatitis. Advantages include its ability to detect subtle and severe changes of the pancreatic duct and parenchyma, and its relative safety compared with endoscopic retrograde cholangiopancreatography. Limitations include inter- and intra-observer variability, operator dependence, and an incomplete understanding of its true accuracy. The Rosemont classification has recently been proposed as a weighted, standardized method that may improve EUS chronic pancreatitis scoring. This paper reviews the published evidence regarding the accuracy of EUS in chronic pancreatitis diagnosis, and enumerates the emerging technologies that have been recently studied which may ultimately improve endosonographic imaging of the pancreas.

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Key words: Chronic pancreatitis; Contrast-enhanced endoscopic ultrasound; Diagnosis; Digital image analysis; Elastography; Endoscopic ultrasound; Pancreatic function testing; Rosemont classification

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INTRODUCTION

Endoscopic ultrasound (EUS) has been used for the diagnosis of chronic pancreatitis (CP) for over two decades. Its primary attribute is its ability to detect mild parenchymal and ductal abnormalities not seen with computed tomography (CT) scans. EUS is of most use in patients with abdominal pain of suspected pancreatic origin and non-diagnostic cross-sectional imaging. In a report entitled "Minimal change chronic pancreatitis", Walsh described 16 patients with typical pancreatic pain and negative or equivocal CT and endoscopic retrograde cholangiopancreatography (ERCP) images[1]. All patients underwent pancreatic resection due to a strong suspicion of CP. Fifteen of the 16 patients had definite histological features of CP, including periductal fibrosis, duct dilation, intralobular inflammation, and atrophy. The histological features were often subtle and mild, out of proportion to the severity of pain. Had EUS been widely available, it might have been used to verify the presence of parenchymal and ductal abnormalities of CP prior to resection. This review focuses on the role of EUS in the diagnosis of CP, including its accuracy, strengths, and limitations. An update on emerging techniques to augment endosonographic diagnosis is also presented.

THE NORMAL PANCREAS

The normal endosonographic appearance of the pan-

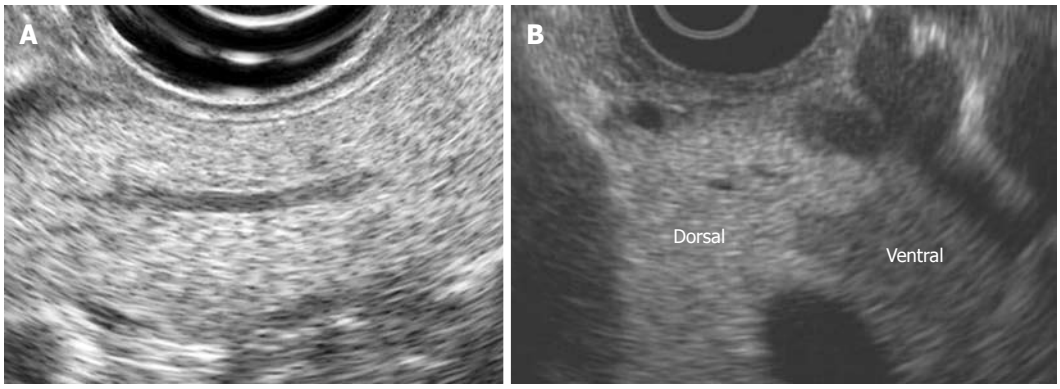


Figure 1 The normal endosonographic appearance of the pancreas. A: View of pancreatic body from gastric station. The parenchyma is homogeneous and granular ("salt and pepper"). The duct is neither dilated nor ectatic; B: Dorsal ventral anlage. The ventral pancreas is relatively echogenic compared with the dorsal pancreas. There is a distinct border between the dorsal and ventral pancreas.

creas has been characterized in three studies of healthy volunteers[2] and patients undergoing EUS for non-pancreatic indications[3,4]. The normal pancreatic parenchyma is homogenous and granular ("salt and pepper echotexture"), and echogenic relative to the liver parenchyma (Figure 1A). The border of the gland is smooth. The main pancreatic duct is also smooth in contour and tapers from the head to the tail. In one study, the upper limit of normal for duct diameter was 3.6 mm in the head, 3.0 mm in the body, and 2.0 mm in the tail[2]. The main pancreatic duct wall is not easily visible, and side-branches are difficult to visualize or narrow in caliber (< 1.0 mm). A ventral anlage is found in one-half of patients (Figure 1B). This is a relative echogenicity of the dorsal compared with the ventral pancreas.

CP CRITERIA

The endosonographic features that have been used for CP diagnosis have evolved since the inception of EUS. In the seminal studies of Dancygier *et al*[5] and Lees[6], a number of parenchymal and ductal features were characterized (Table 1). At that time, EUS diagnosis was qualitative and focused on the global separation of benign from malignant pancreatic diseases[7]. Subsequent studies codified these features into numbered scoring systems[2,5,8]. The most common scoring system includes 5 ductal and 4 parenchymal features (Figures 2 and 3)[8]. The criteria are counted and compared against a cutpoint. Typical cut-points range from 3 (maximizing sensitivity) to 5 (maximizing specificity).

A frequent criticism of standard scoring is that each criterion is counted equally, even though some criteria have more diagnostic importance than others. In April 2007, thirty-two international experts met in Rosemont Illinois, USA to create a consensus-based scoring system[9]. The Rosemont classification includes strictly defined major and minor criteria, and a four-level diagnostic stratification (Table 1). Although this new scoring system may help standardize the EUS diagnosis of CP, it requires further validation in multicenter studies.

TECHNICAL ASPECTS

A complete EUS examination to evaluate CP includes a careful inventory of all major and minor criteria. The body and tail is viewed from the gastric station, where the interpretation of CP criteria is consider most reliable. It is good practice to photograph, digitally annotate, count, and measure all identified features based on the Rosemont definitions. The main pancreatic duct should be carefully traced to ascertain the presence of $\geqslant$ 3 visible side-branches and echogenic wall. The duct diameter should be measured at the genu (in view of the portosplenic confluence) and in the pancreatic tail (in view of the splenic hilum). A 10-s video clip of the body and tail provides a useful frame of reference for future examinations. The scope should be advanced to the duodenum for a careful inspection of the head and uncinate process. Examination of the head detects cysts and main duct calculi (the only criteria properly detected in the head) and rules out biliary pathology, solid masses, and pancreas divisum.

Most validation studies have used mechanical radial scopes for CP diagnosis. However, most current endosonographers prefer linear scopes to examine the pancreas due to higher resolution, color flow Doppler capability, and capability for tissue sampling and celiac plexus blockade. In a blinded study, we found radial EUS to be non-inferior to linear EUS for CP diagnosis[10]. Newer electronic radial scopes have similar resolution and the same Doppler capability as older generation linear scopes.

TEST PERFORMANCE

The ideal study of EUS accuracy might include 1000 consecutive patients with non-diagnostic CT scans from multiple centers undergoing total pancreatectomy for suspected CP. Each patient would undergo a preoperative EUS examination and blinded histological analysis of the resection specimen. Of course, this study is unlikely to ever be performed. Even if it were carried out, some would quibble with the significance of mild or patchy fibrosis on the histological specimen!

Table 1 Evolution of endoscopic ultrasound criteria for chronic pancreatitis

	Lees[6] (1986)	Wiersema *et al*[2] (1993)	Sahai *et al*[8] (1998)	Catalano *et al*[9] (2009)[1]	Histological correlation
Number of criteria	Qualitative	11	9	3 major; 6 minor	
Parenchymal	X	Reduced echogenic foci Heterogeneity	X	X	
	Strongly echogenic foci	Echogenic foci (> 3 mm)	Hyperechoic foci	Hyperechoic foci without shadowing (minor). Echogenic structures ⩾ 2 mm in length and width with no shadowing	Focal fibrosis
				Hyperechoic foci with shadowing (major A). Echogenic structures ⩾ 2 mm in length and width that shadow	Calcifications in side-branches
	Echogenic bands	X	Hyperechoic strands	Stranding (minor). Hyperechoic lines of ⩾ 3 mm in length in at least 2 different directions with respect to the imaged plane	Fibrotic bands
	Cavities	Cysts (> 3 mm)	Cysts	Cysts (minor). Anechoic, rounded/elliptical structures with or without septations	Pseudocysts
	Accentuation of lobular architecture	Accentuation of lobular pattern	Parenchymal lobularity	Lobularity with honeycombing (major B). Well-circumscribed, ⩾ 5 mm structures with enhancing rim and relatively echo-poor center, with ⩾ 3 contiguous lobules	Edema separated by fibrotic bands
				Lobularity without honeycombing (minor). Well-circumscribed, ⩾ 5 mm structures with enhancing rim and relatively echo-poor center, with noncontiguous lobules	
	Enlargement	X	X	X	
Duct	X	Narrowing	X	X	
	Increase in caliber	Dilation	MPD dilation	MPD dilation (minor). ⩾ 3.5 mm in body or > 1.5 mm in tail	Obstructed duct
	Irregularity of the lumen	Irregular contour	Irregular MPD margins	Irregular MPD contour (minor). Uneven or irregular outline and ectatic course	Ductal fibrosis and strictures
	Increase echogenicity of duct wall	Duct wall echogenicity	Hyperechoic MPD margins	Hyperechoic MPD margin (minor). Echogenic, distinct structure greater than 50% of entire MPD in the body and tail	Periductal fibrosis
	Intraluminal echoes	Calculi	Shadowing calcifications	MPD calculi (major A). Echogenic structures within MPD with acoustic shadowing	Calcifications in the main duct
	Visualization of side branches	Side-branch dilation	Visible side-branches	Dilated side branches (minor). 3 or more tubular anechoic structures each measuring ⩾ 1 mm in width, budding from the MPD	Side-branch ectasia
	Strictures with dilation	X	X	X	
	Main or branch duct disruptions with cyst	X	X	X	

[1]Rosemont diagnostic stratification: Normal: < 3 minor features (excluding non-shadowing echogenic foci, visible side-branches, cysts, and MPD dilation) and no major features. Indeterminate: 3 or 4 minor features and no major features, or major B feature alone with < 3 minor features. Suggestive: 1 major A feature and < 3 minor features, or 1 major B feature and ⩾ 3 minor features, or ⩾ 5 minor features. Most consistent: 1 major A feature and ⩾ 3 minor features, or 1 major A feature and major B feature, or 2 major A features. MPD: Main pancreatic duct.

To estimate the test performance of EUS, we must interpret cross-sectional studies with flawed reference standards (e.g. ERCP or functional testing), small histological comparisons with significant selection, spectrum, or sampling biases, and post-EUS observational studies with insufficient follow-up time (Table 2). Sadly, the actual sensitivity and specificity of EUS for diagnosing CP may never be known.

ERCP

The earlier studies compared EUS with ERCP, since ERCP was the *defacto* radiographic reference standard and considered ethical at the time for diagnostic purposes[2-4,8,11]. These studies included performance of both EUS and ERCP in succession, and sometimes blinded independent interpretation of the EUS and ERCP images. Combining 273 patients from the three ERCP studies that used a cutpoint of ⩾ 3 EUS criteria, the sensitivity is 87% and the specificity is 75%[2,3,8]. Limitations of the ERCP reference standard include limited specificity (e.g. in advancing age or following acute pancreatitis), frequent inadequacy of the pancreatogram to interpret small duct changes, and inability to assess the parenchyma[12].

Pancreatic function testing

We might expect high accuracy for EUS in these ERCP comparison studies since one structural test is being compared to another. The comparison of EUS with a pancreatic function test (PFT) reference standard is based on the rationale that impaired exocrine function is a sensitive surrogate for early fibrosis. An unadjusted pooling of the 255 patients in the secretin PFT studies reveals a sensitivity of 72% and specificity of 74%[2,3,13-16], noticeably lower than ERCP studies. A shortcoming of these studies is substantial methodological variation in secretin- and CCK-based PFT protocols. Also, acinar-cell and duct-cell exocrine insufficiency do not perfectly correlate with histological fibrosis. A subset of patients even with advanced structural changes has preserved exocrine function[17].

Histology

Histological comparison may be the "Holy Grail" for

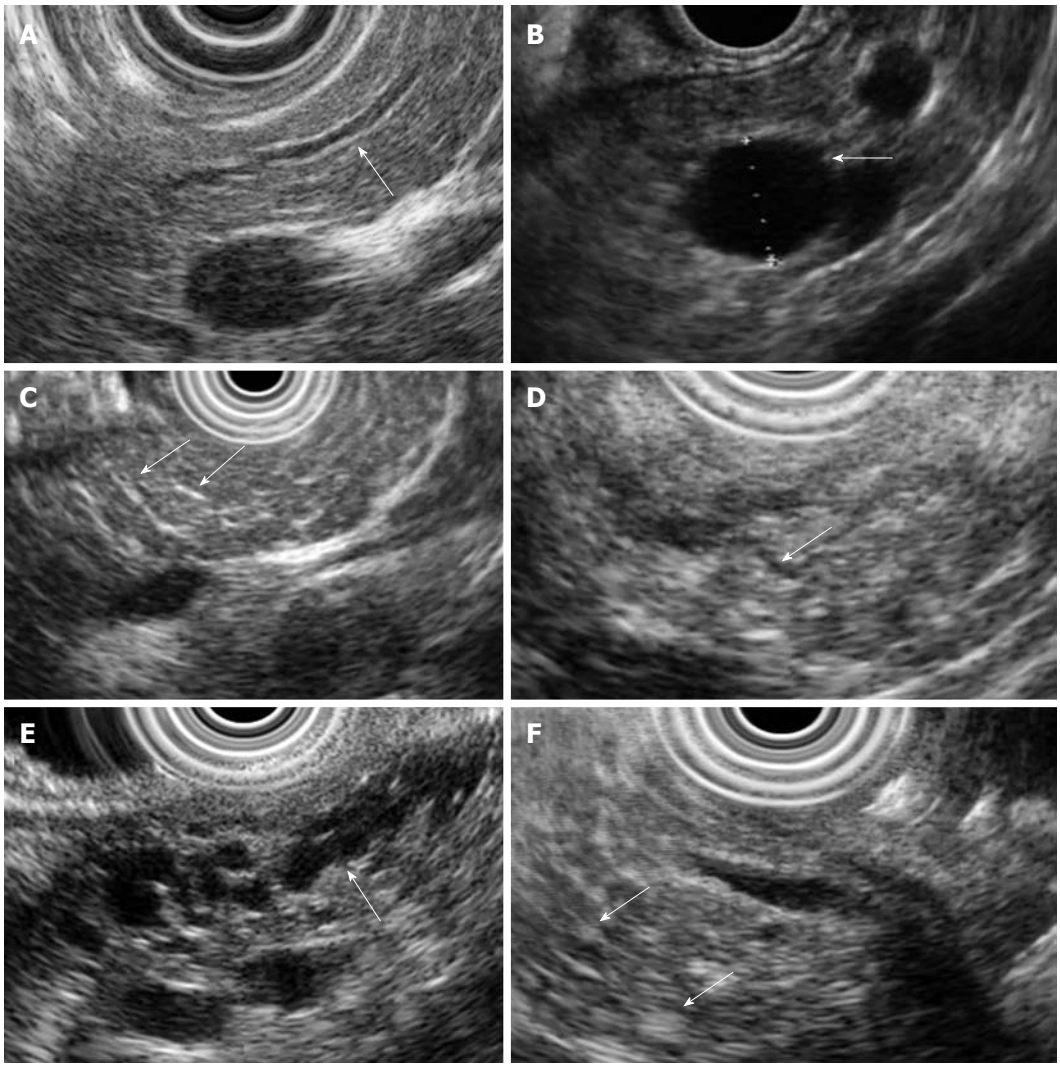


Figure 2 Examples of endoscopic ultrasound (EUS) chronic pancreatitis (CP) criteria. A: Hyperechoic duct wall (arrow); B: Cyst (arrow); C: Hyperechoic strands (arrows); D: Visible side-branch (arrow); E: Dilated and irregular main pancreatic duct with visible side-branches (arrow); F: Hyperechoic foci (arrows).

studying diagnostic tests for CP. There have been four studies comparing EUS with histology, two of which are published in abstract form only[18-21]. Varadarajulu evaluated 42 patients who underwent preoperative EUS within 2 mo of a partial or complete pancreatic resection[18]. Based on an ROC curve analysis, ⩾ 4 criteria optimized sensitivity (91%) and specificity (86%) of EUS. There was a very good correlation of the histological fibrosis score with the EUS score ($r = 0.85$, $P < 0.0001$). A strength of this study was the predominance of mild fibrosis which minimizes spectrum bias. Also, half the patients did not have histological fibrosis allowing a reasonable estimation of specificity. A limitation was that most patients underwent resection for pancreatic cancer, making it difficult to extrapolate the results to the typical patient with abdominal pain.

Chong *et al*[19] reported a larger study in 71 patients with abdominal pain and suspected CP who underwent resection or open biopsy. This study had a substantial proportion of patients with calcific CP. Sixteen of the patients had calcifications on CT; an additional 14 patients had small calcifications detected by EUS. In the 41 patients without calcifications (minimal change group), ⩾ 3 EUS criteria had 83% sensitivity (95% CI: 67%-94%) and 80% (95% CI: 28%-99%) specificity for the diagnosis of CP. The wide confidence limits surrounding the specificity estimate are due to only 5 patients without fibrosis.

These studies are quite helpful in providing histological correlations to validate individual EUS criteria and EUS scoring. One potential limitation of these studies is selection bias since only patients undergoing surgery were included. The increasing performance of total pancreatectomy with autologus islet cell transplant (TP/AIT) for earlier stages of CP may allow future multicenter histological studies in patients with milder fibrosis[20].

Long-term follow-up

Long-term follow-up may be a useful reference standard. Patients with normal and abnormal EUS can be observed to see whether obvious disease progression develops on subsequent imaging tests. This approach may shed light on the predictive value of minimal or mild EUS features. Kahl *et al*[22] followed 32 alcoholic patients with normal baseline

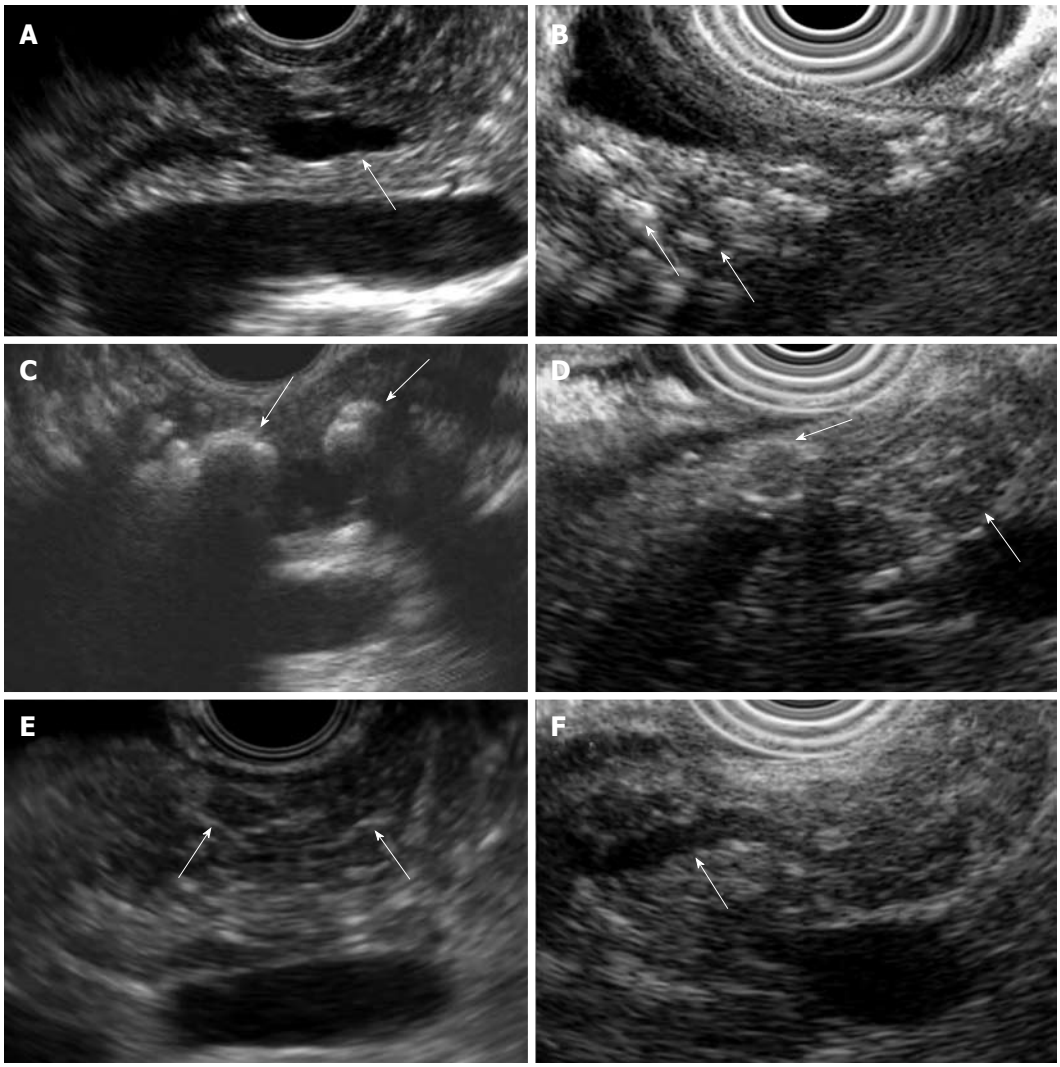


Figure 3 Examples of EUS CP criteria. A: Dilated main pancreatic duct (arrow); B: Parenchymal calcifications (arrows); C: Main duct calcifications (arrows); D: Lobules (arrows); E: Stranding (arrows); F: Irregular main pancreatic duct (arrow).

ERCP and abnormal EUS (≥ 1 criterion) for a median observation time of 18 mo (range 6 to 25 mo). During the follow-up period, ERCP was repeated in 22 patients, and demonstrated either Cambridge Class I (n = 12) or II (n = 10) ductal changes. The authors concluded that mild EUS changes had 100% sensitivity for CP, and predicted the development of CP at an earlier stage than ERCP. The conclusion of this study is based on subtle endpoints of questionable specificity (Cambridge I and II changes). It is not clear whether there were meaningful differences between the baseline and follow-up pancreatograms since interpretation was not blinded. Calcifications and atrophy may be more convincing endpoints, but would require longer follow-up. Natural history studies suggest that calcifications may develop after more than 10 years from symptom onset[23]. Also, Kahl *et al*[22] considered EUS positive if only 1 criterion was present. This cutpoint is likely to result in many false diagnoses. Finally, repeat ERCP was not performed in all patients, which produces verification bias and weakens the estimate of specificity.

Morris-Stiff *et al*[24] identified 16 patients who underwent EUS for evaluation of CP after normal conventional imaging (CT or MRCP). Thirteen of the patients had EUS changes of CP, and all 13 subsequently developed "progression of disease" on subsequent conventional imaging. Neither the follow-up time nor the specific imaging findings referenced as disease progression were reported in this publication.

Hastier *et al*[25] presented results that conflict with Kahl's and Morris-Stiff's findings. In this study, 18 alcoholic cirrhotic patients with isolated parenchymal features identified by EUS and normal ERCP were followed. After a mean duration of 22 mo, the EUS appearance was unchanged in all patients. The subsequent repeat pancreatograms were normal in all 10 patients who had a follow-up ERCP. This study raises doubts over the significance of the importance of minimal EUS features. Since follow-up time was short, firm conclusions cannot be drawn.

In summary, the test performance of EUS can only be estimated based on a variety of imperfect evidence. In interpreting these studies, it is useful to calculate the sensitivity and specificity estimates in the subset of pa-

Table 2 Test performance of endoscopic ultrasound in comparative studies

Study	Gold standard	Cut-point	Overall test performance		Minimal change CP test performance	
			Sensitivity	Specificity	Sensitivity	Specificity
Buscail *et al*[11]	Composite	None	89% (39/44)	100% (18/18)	NR	NR
Nattermann *et al*[4]	ERCP	None	98% (50/51)	63% (27/43)	88% (7/8)	63% (27/43)
Wiersema *et al*[2]	ERCP	≥ 3	100% (19/19)	79% (38/48)	100% (18/18)	79% (38/48)
Catalano *et al*[3]	ERCP	≥ 3	86% (31/36)	95% (42/44)	82% (22/27)	95% (42/44)
Sahai *et al*[8]	ERCP	≥ 3	63% NR	68% NR	NR	NR
Hastier *et al*[25]	ERCP	None	93% (13/14)	NR	NR	NR
Hollerbach *et al*[36]	ERCP	Grade 0-3	97% (30/31)	67% (4/6)	95% (20/21)	67% (4/6)
Wiersema *et al*[2]	Secretin ID-PFT	≥ 3	67% (6/9)	29% (2/7)	67% (6/9)	29% (2/7)
Catalano *et al*[3]	Secretin ID-PFT	≥ 3	84% (21/25)	78% (43/55)	NR	NR
Raimondo *et al*[13]	CCK PFT	≥ 4	43% (3/7)	50% (4/8)	NR	NR
Chowdhury *et al*[14]	Secretin PFT	≥ 3	71% (5/7)	35% (5/14)	71% (5/7)	35% (5/14)
Stevens *et al*[15]	Secretin PFT	≥ 3	68% (34/50)	79% (30/38)	NR	NR
Stevens *et al*[16]	Secretin PFT	≥ 4	71% (17/24)	92% (24/26)	NR	NR
Stevens *et al*[16]	CCK PFT	≥ 4	63% (15/24)	85% (22/26)	NR	NR
Chong *et al*[19]	Histology	≥ 3	NR	NR	83% (30/36)	80% (4/5)
Varadarajulu *et al*[18]	Histology	≥ 4	91% (19/21)	86% (18/21)	91% (19/21)	86% (18/21)
Gupta *et al*[20]	Histology	≥ 3	71% (10/14)	100% (1/1)	71% (10/14)	100% (1/1)
Albashir *et al*[21]	Histology	≥ 4	75% (12/16)	NR	NR	NR
Bhutani *et al*[49]	Histology (Autopsy)	≥ 3	100% (10/10)	100% (1/1)	100% (10/10)	100% (1/1)
Kahl *et al*[22]	Baseline or repeat ERCP	≥ 1	100% (114/114)	NR	NR	NR
Morris-Stiff *et al*[24]	Radiographic progression	None	100% (13/13)	NR	100% (13/13)	NR

Calc: Calcification; CCK: Cholecystokinin; NR: Not reported or calculable; PFT: Pancreatic function test; ID-PFT: Intraductal pancreatic function test.

tients with non-calcific (minimal change) CP, where EUS is of most clinical value (Table 2). At the present time, we must take a "nihilistic" view of the diagnostic performance of EUS accuracy and interpret the results with care in the context of the history and ancillary laboratory, imaging, and functional test results.

RELIABILITY

The major strengths of EUS are its ability to detect mild structural abnormalities of the entire pancreas and its safety relative to ERCP. Unfortunately, there are also several caveats.

Questionable specificity

Minimal or mild parenchymal and ductal EUS features may have poor specificity, resulting in the "overdiagnosis" of CP. A number of clinical variables can lead to EUS changes that mimic those in CP. Rajan performed a prospective study of 120 patients undergoing EGD/EUS for non-pancreatic indications[26]. At least 1 EUS CP criterion was found in 38% of these non-alcoholic and asymptomatic patients. Patients in the > 60 years age group had a statistically insignificant trend toward more frequent EUS abnormalities (39%) compared with those in the < 40 years (23%) and 40-60 years (25%) age groups (P = 0.13). Male gender was a statistically significant predictor of an abnormal EUS (OR 2.9, 95% CI: 1.2-6.8).

In another study, 189 asymptomatic patients undergoing EUS for non-pancreatic indications were administered an alcohol questionnaire[27]. EUS features were more frequent in alcoholic patients (70%) compared with non-alcoholic patients (31%) (P < 0.001), suggesting the EUS changes may be present in alcohol drinkers who lack symptoms of CP. In a much larger study (n = 1157), heavy ethanol intake (OR 5.1, 95% CI: 3.1-8.5), male sex (OR 1.8, 95% CI: 1.3-2.6), clinical suspicion of pancreatic disease (OR 1.7, 95% CI: 1.2-2.3), and heavy smoking (OR 1.7, 95% CI: 1.2-2.4) were each independent predictors of severe EUS changes[28].

A fatty pancreas causes hyperechogenicity of the parenchyma that can imitate the echogenic changes of CP. In a logistic regression analysis, obesity, fatty liver disease, and alcohol intake were independent predictors of fatty infiltration of the pancreas[29]. Another study showed a high rate of EUS abnormalities in patients with dyspepsia (and low suspicion of pancreatic disease) compared with controls[30]. This may indicate that mild CP is a more common etiology of dyspepsia than previously thought. Other plausible explanations include tertiary referral bias and information bias, since the endoscopist was aware of the clinical history during the EUS examination[31]. In summary, age, gender, smoking, alcohol use, and BMI may influence EUS results. Future scoring systems may include these clinical variables in addition to EUS features.

Interobserver variability

Interobserver variability pertains to differences in interpretation of the same exam by multiple observers. In a study by Wallace, 45 videotaped examinations were privately scored by 11 expert endosonographers[32]. The κ (agreement beyond chance) for global diagnosis was only moderate (κ = 0.45). Interobserver agreement was worse (κ < 0.40) for most individual criteria, except for lobularity (κ = 0.51) and main duct dilation (κ = 0.61). Similar sub-optimal interobserver agreement was observed

in a study of CP criteria in familial pancreatic cancer kindreds ($\kappa < 0.40$ for all CP criteria except cysts)[33].

It was expected that the Rosemont classification might improve interobserver agreement because it incorporates more stringent definitions. In a recent study, 50 clips were interpreted by 14 experts on two occasions, first with standard scoring and then with Rosemont scoring[34]. The interobserver agreement was slightly higher for Rosemont scoring [$\kappa = 0.65$ (95% CI: 0.52-0.77)] compared with standard scoring [$\kappa = 0.54$ (95% CI: 0.44-0.66)]; however, the difference was not statistically significant ($P = 0.12$).

Intraobserver variability

Intraobserver variability relates to differences in scoring of the same images from multiple interpretations by the same observer. A single study has examined the intraobserver variability of EUS CP criteria[35]. Thirty still-frame images were scored by the same observers on repeat occasions over 6 wk. The intraobserver κ for overall diagnosis of CP was 0.77, and ranged from 0.61 to 0.88 for individual criteria. In other words, endosonographers have reasonable (but not perfect) agreement with themselves when interpreting the same image on multiple occasions.

Pretest probability

EUS is known as an operator dependent test. Even with Rosemont scoring, there is inherent subjectivity in measuring each criterion. Most endosonographers are aware of the patient's history as they are performing the examination, and may have already formed a "likelihood of disease" estimate in their mind. This pre-test probability may influence the interpretation of EUS results. For example, in a non-smoker with dyspepsia and no history of heavy alcohol intake or acute pancreatitis, many endosonographers would ignore mild stranding and lobularity in fear of mislabeling the patient. There are no available studies characterizing the affect of pretest probability on EUS interpretation.

ANCILLARY TECHNIQUES

Several supplementary techniques have been studied which may complement standard EUS scoring in the diagnosis of CP.

Tissue sampling

Linear EUS allows tissue acquisition through fine needle aspiration (FNA). Hollerbach *et al*[36] reported 27 patients with suspicion of CP who underwent targeted fine-needle aspiration of lobular or inflamed appearing areas of the pancreas. An average of 2.3 passes was required to obtain satisfactory tissue in all patients. The cytological findings were graded 0 to 2 based on the severity of chronic inflammation. The performance of FNA minimally increased the specificity of EUS (67% with FNA, 60% without FNA). Two patients developed mild acute pancreatitis following the procedure. Based on this study, routine FNA does not seem very useful given its minimal impact on test performance, potential for acute pancreatitis, and the lack of a validated cytological grading system.

Obtaining a core biopsy may provide sufficient tissue for a histological diagnosis. Dewitt performed EUS-guided Trucut biopsy (TCB) of the pancreatic body in 16 patients with suspected non-focal CP[37]. Only 1 patient had CP based on the histological analysis; 8 had normal pancreas; 6 had non-diagnostic findings; 1 had device malfunction. Interestingly, all of the patients with normal pancreatic histology had 3-5 features on EUS imaging. Most of the patients with non-diagnostic histopathology had severe ($\geqslant 5$) EUS features, with only connective tissue apparent on the pathologic specimen. There were 2 hospitalizations for post-procedural abdominal pain and pancreatitis. The authors concluded that EUS-TCB was not recommended based on low yield and risk of complications. However, a more favorable interpretation is that TCB lowers the number of false positive EUS results.

Digital image analysis

Digital image analysis (DIA) limits operator dependence by trading human interpretation for computerized pattern recognition. The colors, distribution, and patterns of individual pixels are algorithmically quantified within a specified region of interest. Irisawa *et al*[38] digitally quantified the parenchymal echogenicity in CP patients and controls. The mean parenchymal echogenicity was determined as the amount of field area over a threshold level of echogenicity. The mean size above the threshold area was significantly higher in CP patients compared to controls. Also, echogenicity correlated with disease severity.

In another study, a sophisticated program was used to compare images from patients with CP, pancreatic cancer, and controls[39]. A total of 256 statistical parameters were extracted from each region of interest and filtered into a compact dataset of 11 textural parameters. These 11 variables were used to train and validate a neural network model for interpretation of each image. The end model was 100% accurate in classifying normal pancreas from CP. Further refinement of existing software is required to make DIA an easy and reliable method for real-time image interpretation.

Spectrum analysis

B-mode ultrasound gray-scale images are derived from the amplitude of reflected radiofrequency waves. Additional information can be gained from the spectrum of hidden back-scattered radiofrequency signals. This frequency information is typically ignored and not used to generate the image. However, backscattering is related to the density and sound speed of the tissue, and may represent a useful parameter to quantify pancreatic fibrosis. In a pilot study, statistically significant differences in spectral parameters computed from the backscatter were observed between normal pancreas and CP[40].

Elastography

A method to measure the "hardness" of tissue may be

very applicable for early CP diagnosis. Tissue elastography assesses tissue stiffness by measuring the response of the tissue to mechanical compression. As the scope gently compresses the tissue, less tissue displacement occurs with hard relative to soft tissue. This displacement (or "strain") can be quantified, color coded, and superimposed over the B-mode image.

There have been two initial reports of EUS elastography for CP. Janssen compared the elastography images in controls, patients with CP, and patients with pancreatic masses[41]. A "honeycombed" elastography pattern was apparent in most of the CP patients and patients with pancreatic masses. Most of the controls lacked the honeycombed appearance. These results suggest that elastography is useful in separating normal pancreas from CP, and less helpful in differentiating benign from malignant masses.

Săftoiu *et al*[42] applied sophisticated image analysis to the interpretation of EUS elastography. Mean hue histograms were calculated for regions of interest from controls, inflammatory masses, and malignant masses. Receiver operating characteristics (ROC) analysis revealed that a mean hue histogram cutpoint of 175 had 91.4% sensitivity and 89.9% specificity for diagnosis of benign from malignant masses. Neural network analysis of the images was employed to refine the diagnostic performance. After training the neural network, accuracy was as high as 97%.

Although initial results with elastography and other image analysis approaches are encouraging, many questions remain. Do these new techniques improve upon standard EUS scoring? Are they easily applied in real time? How much does operator dependence affect the results? It is worth noting that many of these pilot studies have not incorporated blinded interpretation of the images.

Contrast-enhanced EUS

Venous contrast agents may improve EUS resolution of pancreatic tissue and help differentiate benign from malignant masses. In one study, injection of the contrast agent Albumex produced enhancement in 3 of 4 patients with inflammatory masses *vs* 0 of 11 patients with pancreatic cancer[43]. In a study of 86 patients with benign and malignant masses, contrast enhancement improved sensitivity from 73.2% to 91.1%, and specificity from 83.3% to 93.3%[44]. There are no published studies of contrast-enhanced EUS for the diagnosis of early CP.

Endoscopic pancreatic function testing

Some experts suggest that both structural and functional testing is needed to optimally diagnose early CP[45]. Mild exocrine insufficiency detected by direct pancreatic function tests is a surrogate marker for early fibrosis. Duodenal aspirates can be collected through the endoscope following secretin stimulation, and analyzed for bicarbonate as a measure of duct-cell exocrine function. This endoscopic pancreatic function test method (ePFT) has shown equivalent bicarbonate concentration results as the traditional "Dreiling tube" secretin pancreatic function test in a crossover study[46]. We have recently combined EUS and ePFT in a single 50-min endoscopic session to allow a simultaneous assessment of pancreatic structure and function[16,47]. Concordant normal results (< 4 EUS criteria and peak bicarbonate concentration ⩾ 80 mmol/L) rule out CP; concordant abnormal results (⩾ 4 EUS criteria and peak bicarbonate concentration < 80 mmol/L) rule in CP. The greater diagnostic challenge arises when the structural and function results are discordant. Long-term observational studies are underway to clarify the relative importance of early structural and functional changes in predicting early fibrosis. In the meantime, the EUS and ePFT results should be interpreted in the context of the patient's symptoms and risk factors.

Secretin enhanced EUS

Secretin may also be used to "dynamically" image the pancreatic duct in patients with suspected CP. Catalano *et al*[48] serially measured the pancreatic duct following secretin injection in 40 patients evaluated for suspected CP. Secretin-induced duct dilation was 80% sensitive and 96% specific for detecting ductal obstruction (stones and strictures, and also predicted response to minor papillotomy in patients with pancreas divisum.

CONCLUSION

Endoscopic ultrasound is a useful and safe technique for detecting pancreatic parenchymal and ductal abnormalities suggestive of chronic pancreatitis. Even after many comparative studies, the accuracy of EUS for diagnosing early chronic pancreatitis is poorly understood. In spite of this and other limitations, many gastroenterologists consider EUS the best available structural reference standard. It is hoped that emerging ancillary technologies will further refine the EUS diagnosis of chronic pancreatitis in coming years.

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Fixation methods for electron microscopy of human and other liver

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Abstract

For an electron microscopic study of the liver, expertise and complicated, time-consuming processing of hepatic tissues and cells is needed. The interpretation of electron microscopy (EM) images requires knowledge of the liver fine structure and experience with the numerous artifacts in fixation, embedding, sectioning, contrast staining and microscopic imaging. Hence, the aim of this paper is to present a detailed summary of different methods for the preparation of hepatic cells and tissue, for the purpose of preserving long-standing expertise and to encourage new investigators and clinicians to include EM studies of liver cells and tissue in their projects.

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Key words: Liver; Tissue fixation; Perfusion; Electron microscopy; Biopsy

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INTRODUCTION

For an electron microscopic study of the liver, expertise and complicated, time-consuming processing of hepatic cells and tissues is needed. The interpretation of electron microscopy (EM) images requires knowledge of the liver fine structure and experience with the numerous artifacts in fixation, embedding, sectioning, contrast staining and microscopic imaging. In addition, the considerable investment in equipment and the costs of personnel, together with the construction and maintenance of dedicated laboratory space and accessory equipment, makes EM investigation of cells and tissues one of the most cost-intensive approaches in biomedical research. The choice of the

right preparation method and the use of good criteria to evaluate the quality of the preparation, together with the right interpretation of the results, is crucial not only for getting the best out of the method, but also to avoid misuse of tax payers money. With sadness, we conclude that the old trade of traditional EM studies is disappearing, and is being partly replaced by immunological techniques and confocal or fluorescence microscopy[1,2]. Hence, the aim of this special paper is to present a detailed summary of different methods for the preparation of hepatic cells and tissue, for the sake of preserving long-standing expertise and to encourage new investigators and clinicians to include EM studies of liver cells and tissue in their projects. In a recent review and guidelines on the clinical use of liver needle biopsies by Rockey *et al*[3], it is stated that, at present, there is only a minor or even negligible role for the use of EM in the diagnosis, prognosis and management of patients with liver disease.

METHODS OF FIXATION AND PREPARATION

Fixation has several goals: (1) to stop metabolism; (2) to fix structures of organelles and molecules in their current position; and (3) to make material accessible and stable during further processing. When metabolic activity in tissues is not arrested, autolytic changes will develop within minutes or even seconds. The need to use extremely fresh tissue, preferably without touching and deformation is therefore of utmost importance. The fixation is also responsible for crosslinking and arresting many molecules with the result that cells, organelles and their substructures are preserved. After embedding, sectioning and contrast staining, these structures become visualized in the electron microscope at magnifications in the range of 50 to 1 000 000 times. The protocols given hereafter can be used for light microscopy, scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

In a situation where experimental animals are used, the method of perfusing a fixative through the portal vein is by far the best method to choose[4-7]. When a complete liver is not available, injecting the fixative into a piece of tissue not smaller than 1 cm provides comparable results to portal vein perfusion. This method can be applied to experimental or clinical wedge biopsies[8-13]. An advantage of this method is the fact that it only requires a small opening of the peritoneal wall to collect a small piece of tissue. With regard to needle biopsies, there have been attempts to inject the fixative into the tissue of the biopsy, with quite good results[8,10,12,14-16]. Unfortunately, these attempts have not resulted in routine applications and immersion fixation remained the standard method of fixing liver needle biopsy specimens. Vascular perfusion through a partly resected liver not used for transplantation, has also been reported to be successful[17]. In order to complete the series of protocols, we have added a short description of fixation of liver cell cultures.

Important elements in fixation are the chemical composition of fluids and the physical conditions during their application. Fluids involved in the fixation process should be isotonic to the cells; in other words, the fixation fluid should be as physiological as possible, with the exception of the fixing compound[18]. To avoid temperature shock of cells and tissues before the actual fixation sets in, isothermic fluids should be used in principle. The major condition for fixation is to transfer cells and tissues from their natural environment to the fixing environment, as rapidly as possible: this should take not more than a few seconds, be it in the operating room or in the laboratory. Living cells and tissues should not be left alone, not even for a few seconds, when this can be avoided. One should avoid subjecting the cells or tissue to drying or dry conditions, keep them wet and well-immersed in fluid, and watch the meniscus carefully to ensure that it does not touch the cells, because it will destroy them.

Fixation should be applied at the cellular level, i.e. bring all cells instantaneously and simultaneously into contact with the fixative. Immersion fixation of small pieces of tissue by plunging them into a fixative has to be avoided as much as possible. The slow penetration of a fixative into a 1-mm piece of tissue results in bad fixation in the center of the block. During the penetration of the fixative, its constituents react differently with cellular components, thereby changing the composition of the fixative on its way to the center of the block. The vehicle (buffer) is supposed to penetrate more rapidly, at the same time slowing down the fixing compound. Immersion fixation might leave the center of a tissue block unfixed for the major part of an hour. Nevertheless, immersion-fixed liver biopsies might provide sufficient information on the ultrastructure of parenchymal cells, but unfortunately sinusoids and sinusoidal cells are destroyed by immersion fixation.

Perfusion protocols allow a regimen of different fluids to be flushed through the liver[18]. Since osmium is an expensive and very toxic substance, it is not recommended to apply this fixative by perfusion. We have tried osmium perfusion[4], but the results were poor[18]. For post-fixation, the tissue should be cut into pieces that are washed in buffer to avoid chemical reactions between glutaraldehyde and osmium. The tissue blocks are subsequently immersed in the buffered osmium tetroxide. By doing so, one has control over a toxic procedure, while there is free admission of osmium to the cells through open veins and sinusoids, and the rapid application of a second fixative with a different reaction mechanism[19]. Although ethanol is used to dehydrate the tissue, its capacity to precipitate proteins and harden the tissue contributes to the fixation process. In order to better preserve membranes and their contrast, several additives to the fixatives have been proposed and applied, such as Ca^{2+} or Li^{+} ions, ferrocyanide[20], tannic acid, and picric acid[19,21].

Cryotechniques, molecular identification of cellular components, and their three-dimensional reconstruction or cryofracture as an alternative method to chemical fixation are not the subject of this review.

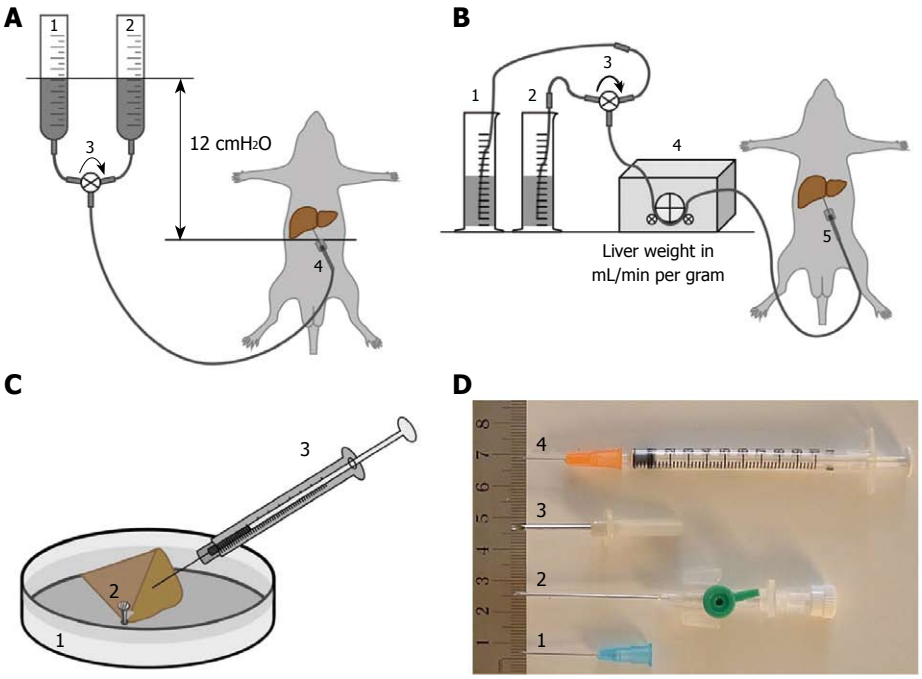


Figure 1 Diagram schematically depicting the different and most frequently used perfusion-fixation methods, including perfusion-fixation needles, to fix hepatic tissue. A: Gravity-mediated perfusion fixation using 12 cm water pressure: (1) pre-perfusion buffer; (2) fixative solution; (3) tri-valve system (e.g. Discofix® C, B. Braun, Switzerland) that facilitates the change-over between both solutions; and (4) cannulation of the portal vein; B: Roller pump-mediated perfusion fixation at a flow of 1 mL/g liver tissue: (1) pre-perfusion buffer; (2) fixative solution; (3) tri-valve system; (4) low-flow peristaltic pump; and (5) cannulation of the portal vein; C: Injection or puncture perfusion fixation, using a needle to inject fixative into a wedge biopsy of about 1 cm × 1 cm × 1 cm dimensions. Injection to approach a rate of 1 mL/min: (1) Petri dish filled with 37℃ physiological saline solution; (2) liver tissue wedge fixed by the tip of a forceps; and (3) syringe containing fixative solution; D: Different types of needles and their variants commonly used for portal vein insertion: (1) 18-23 G needle typically used for gravity-mediated perfusion for rat and mouse livers; (2) 18 G needle catheters are recommended for peristaltic-pump-mediated perfusion; (3) 12-13 G needles are typically used for larger animals such as rabbits; and (4) 25 G syringes are ideal for the injection of fixative solution in hepatic tissue. In case of accidental failure of procedure A or B, method C (injection perfusion) can be used to save the preparation.

FIXATION PROTOCOLS

Perfusion fixation of total liver in situ (mostly applied to experimental animals)[5,6,22,23]

The procedure for perfusion fixation of total liver *in situ* is as follows: (1) Anesthetize the animal, preferably with 4.5 mg/100 g body weight Nembutal (which also relaxes the musculature); (2) Fix the animal to a waterproof surgical support with its back down; (3) Shave and disinfect the animal's abdomen; (4) Open the abdominal cavity along the linea alba, with lateral cuts along the ribs and lower segment; (5) Gently move the intestines aside and cover them with surgical cotton or bandages wetted with warm physiological saline to keep them moist and warm (one could also use an infrared lamp); (6) Expose the portal vein and prepare separate, double ligatures around it; (7) Take care that the hepatic artery is included in the ligature; (8) Introduce the largest possible (just fitting) needle into the portal vein, after connecting it to a silicon tube that is connected to the perfusion system (fluids on room temperature, glass vessels or peristaltic pump, Figure 1A and B); (9) Constrict the ligatures independently, taking care not to puncture the portal vein, and make sure to close to the hepatic artery; (10) Start perfusion with glutaraldehyde solution by opening a valve or switching on the peristaltic pump; the flow rate in mL/min should be more or less equal the total weight of the liver in grams; (11) Incise the vena cava to allow fluids to escape from the vascular system, and aspirate fluids when necessary; (12) Watch the liver change in color and consistency. This process should start within the first minute (typically 15-30 s); (13) Stop the perfusion after 5 min; (14) Gently remove the liver or one or two well-perfused lobes and put them into a Petri dish that contains fixative. Well-perfused liver lobes change their color from dark red to yellow/brown, whereas the consistency changes from soft to hard like a boiled egg. Badly perfused parts of the liver that are entirely or partly soft and still dark red-brown in color should not be processed. Note that some lobes, e.g. the caudate lobe, often show better perfusion than other ones; (15) Clean a razor blade with ethanol and paper tissue (to remove protecting grease) and gently cut 1-mm slices of liver tissue. Do not put any pressure on the tissue while cutting, and make sawing movements with the razor blade; (16) Keep tissue slices covered with glutaraldehyde and cut multiple 1 mm × 1 mm strips under fluid; (17) Cut several strips simultaneously into 1 mm × 1 mm × 1 mm blocks for TEM, or 1 mm × 1 mm × 5 mm strips for SEM and/or 5 mm × 5 mm × 1 mm slices for light microscopy (LM) flat embedding; (18) Total time in glutaraldehyde should not be longer than 20 min; (19) Transfer blocks to washing buffer (which is the buffer of the glutaraldehyde fixative) to remove glutaraldehyde before contact with osmium; (20) Transfer blocks to 1% buffered osmium in small glass vessels and close these with a cap; (21) Postfix for 1 h in osmium, keep the small vessels at 4℃, and shake

Table 1 Dehydration and embedding for TEM and SEM

TEM		
Ethanol 70%	15 min	20℃
Ethanol 90%	15 min	20℃
Ethanol 100%	30 min	20℃
Ethanol 100%	30 min	20℃
Ethanol 100%/Epon 1/1	Overnight	20℃
Epon	1 h	20℃
Epon 1 (overnight)	BEEM capsules on top	20℃
Epon	Fill BEEM capsules	20℃
Oven	67 h	40-60℃
SEM		
Ethanol 70%	15 min	20℃
Ethanol 90%	15 min	20℃
Ethanol 100%	30 min	20℃
Ethanol 100%	30 min	20℃
Ethanol 100%	Overnight	20℃
Freeze and fracture	Liquid nitrogen	-196℃
Ethanol 100%	30 min	20℃
Critical point drying, phase 1	CO_2/ethanol	16℃
Phase 2 or HDMS drying (see below)	80 bar	35℃
Sputter coating	Pt/Au	

Cutting of ultrathin sections on an ultramicrotome, contrast staining with lead and uranyl salts. TEM: Transmission electron microscopy; SEM: Scanning electron microscopy.

the fluid gently and regularly; (22) Transfer the tissue blocks to the second washing buffer (osmium-vehicle buffer), and wash once or twice to remove osmium; (23) Transfer the blocks to 70% ethanol (v/v); (24) Change 70% ethanol three times; and (25) Transport in 70% ethanol or follow the protocol for further dehydration in ethanol and embedding or critical point drying (see common trunk) (Table 1).

For a summary of necessary chemicals and solutions, composition of fluids, see Table 2.

Fixation of liver wedge biopsies (mostly applied to human liver tissue) (Figure 1C)

Wedge biopsies of roughly 1 cm × 1 cm × 1 cm (or less), including the Glisson's capsule at two sides of the wedge, are taken from the margin of a liver lobe as soon as the surgeon/operator has access to the liver. The operator should be cooperative in order to assure that freshness of the tissue is an absolute priority. Directly after opening of the abdomen *via* a chevron incision, the liver is visualized and a wedge is taken with a sharp scissor from the edge of segment 5 or 6, without manipulating the tissue. Bleeding at the biopsy site can easily be controlled using diathermia or argon laser. In a consecutive series of 150 patients no rebleeding at biopsy sites was reported (unpublished results). (1) The tissue is immediately transferred to a 50-mL vessel, filled with PBS (pH 7.4) at 37℃; (2) The tissue is transported to an annex to the operation theater, where injection perfusion with glutaraldehyde fixative is performed in a Petri dish filled with saline; (3) The wedge biopsy is held at a corner by a forceps and is injected by a 25 G syringe from multiple sides with 1.5% glutaraldehyde fixative, until a discoloration and hardening of the tissue, comparable to total liver perfusion, is obtained; (4)

Table 2 Addendum summarizing chemicals necessary for fixation of liver tissue

Compound	Quantity
Cacodylate buffer 0.2 mol/L, pH 7.4	700 mL
Sodium cacodylate trihydrate (Sigma, C0250)	22.9 g
MQ-(purified by ion-exchange)-water	674.8 mL
HCl (0.2 mol/L)	25.2 mL
Filter through 0.2 μm Millipore or Wattman filter paper	
Cacodylate buffer 0.15 mol/L, pH 7.4	
Cacodylate buffer 0.2 mol/L	900 mL
MQ-water	300 mL
Filter through 0.2 μm Millipore or Wattman filter paper	
Glutaraldehyde fixative, 1.5% in cacodylate buffer 0.067 mol/L	
Cacodylate buffer 0.2 mol/L	575 mL
Sucrose (Sigma, S0389)	17.215 g
Glutaraldehyde solution 70% (Sigma, G7776)	36.96 mL
MQ-water	1113.04 mL
Filter	
Osmium tetroxide 1% in phosphate buffer[1]	90 mL (6 × 15 mL)
Osmium tetroxide 4% for EM (Sigma, 75632)	22.5 mL
Phosphate buffer 0.2 mol/L	45 mL
MQ-water	22.5 mL
Washing buffer 1	
Cacodylate buffer 0.2 mol/L	30 mL
Sucrose	0.9 g
MQ-water	60 mL
Washing buffer 2	
Phosphate buffer 0.2 mol/L	45 mL
MQ-water	45 mL
Phosphate buffer 0.2 mol/L pH 7.4 (200 mL)	200 mL
0.2 mol/L $Na_2HPO_4.2H_2O$ (17.8 g in 500 mL MQ-water)	162 mL
0.2 mol/L $NaH_2PO_4.H_2O$ (13.8 g in 500 mL MQ-water)	38 mL

Fluids and chemicals (volumes to be adapted to needs). [1]An alternative to the osmium postfixation is addition of 1.5% potassium ferrocyanide to 1% osmium in veronal acetate buffer (pH 7.4) at 4℃ for 2 h, which strongly enhances the contrast of membranes in cells and tissues[20] (see Figure 13).

Care should be taken that the injection needle does not operate as a biopsy needle; it should be and stay completely filled with glutaraldehyde during injection into the tissue; (5) After injecting the needle into the tissue, parallel to the capsule rim, the needle should be withdrawn a few millimeters before starting injection of the fixative fluid. The idea is to create a space within the tissue in which the fixative can spread out; (6) Injections should be repeated several times until the blood is flushed out of the tissue and the consistency has changed to that of a hard-boiled egg; (7) The time between excision of the wedge and perfusion with fixative is critical. Total glutaraldehyde time (before washing and before osmication) should not exceed 20 min; and (8) After perfusion with glutaraldehyde, the biopsy is rapidly transported to the laboratory, where slices (1 mm thick), strips (1 mm × 1 mm × 5 mm), small flat slices (5 mm × 5 mm × 1 mm) and tissue blocks (1 mm × 1 mm × 1 mm) can be cut under fluid with a clean, sharp razor blade.

Immersion fixation of liver needle biopsies (Figure 1D)

The percutaneous liver biopsy procedure starts with local anesthesia with 1% lidocaine. Under real-time ultrasound guidance, the liver and the biopsy needle are imaged throughout the procedure. The biopsy is taken by a cut-

ting-type needle and a spring-loaded device. A 16-gauge needle is placed into the liver parenchyma before the device is triggered. This results in a liver specimen of 1-2 cm in length, with the diameter being dependent on the size of the biopsy needle (1-1.8 mm). After collecting the biopsy, the fresh tissue is placed in a Petri dish in which 3 mm × 1 mm × 1 mm pieces of the ends of the cylinder are cut. The remaining tissue is used for diagnostic purposes in the pathology department and separately undergoes routine pathology fixation. This procedure should not take longer than 30 s. The two cylinder-shaped tissue pieces of 3 mm × 1 mm are covered with fixative, cut into 1 mm × 1 mm × 1 mm pieces and transferred to a 15-mL vessel that is filled with 1.5% glutaraldehyde fixative at room temperature. The vessel is gently shaken a few times. The total glutaraldehyde time (before washing and osmication) should be no less than 1 h, in order to allow the fixative to penetrate the tissue. After glutaraldehyde, the tissue will be further processed in washing buffer, osmium fixative, and ethanol dehydration.

Fixation of liver cell cultures

The procedure is as follows: (1) cells are cultured on 1 cm diameter glass or plastic coverslips (0.17 mm thick) in wells of culture dishes; (2) cultures are washed three times with 37℃ buffer to rinse away dead cells, cellular debris and precipitates; (3) culture medium is replaced with glutaraldehyde fixative; (4) one should take care that the cells are always covered by a layer of fluid, so that the meniscus does not touch the cells; (5) fluids are changed several times to ensure complete replacement; (6) wash in cacodylate buffer; (7) postfix in 1% osmium tetroxide in phosphate buffer; (8) wash in phosphate buffer; and (9) dehydrate by starting with 70% ethanol (Table 1, Figures 2 and 3).

Critical point drying (CPD) is used routinely to dry samples for SEM[24]. Nation[25] has reported an alternative method that consists of air-drying of insect tissues by rapid evaporation of hexamethyldisilazane (HMDS), which has a very low surface tension. This is supposed to avoid fracturing and destruction of the specimen during drying. In spite of the advantages of HMDS, such as the absence of high pressure equipment, CPD seems to be the most frequently used method. Drying of biological samples by CPD also causes a shrinkage of about 30% of the volume[26]. We have routinely and successfully applied HMDS to cultured liver cells[27]. HMDS drying takes only 3 min instead of the laborious and time-consuming CPD process. We also have applied HMDS drying to perfusion-fixed liver tissue, and during a comparison with CPD, no differences could be seen between the two methods (Figure 3).

After 100% ethanol, samples are immersed for 3 min (cell cultures) or 10 min (liver tissue) in 100% HMDS. After HMDS, the samples are removed from the culture wells and excess HMDS is blotted away by filter paper. The samples are then transferred to a desiccator for 25 min to avoid water contamination. After drying, the samples are mounted on stubs (Figures 4 and 5) and are sputter-coated with 10 nm gold and made available for SEM investigation.

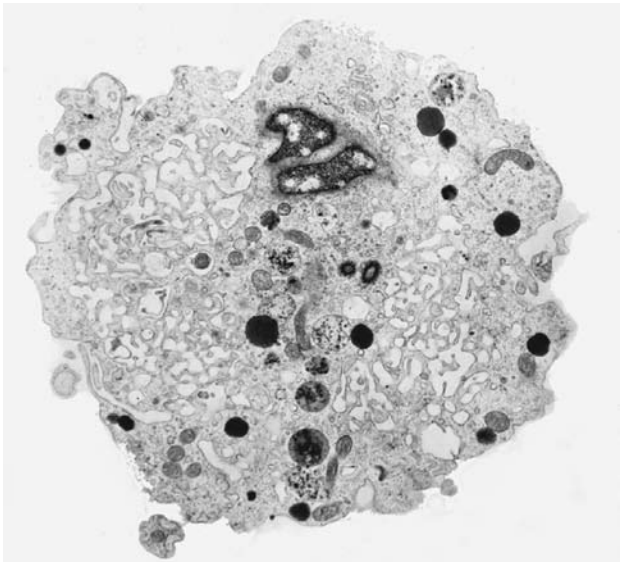


Figure 2 Transmission electron microscopy (TEM) image of an ultrathin section of a plastic-embedded liver sinusoidal endothelial cell after isolation. In the upper part of the picture is a small portion of the nucleus. The labyrinth-like structures in the cytoplasm represent fenestrae that are internalized during the isolation procedure. Electron-dense granules represent lysosomes that reflect the high digestive capacity of these cells. Magnification 6300 ×.

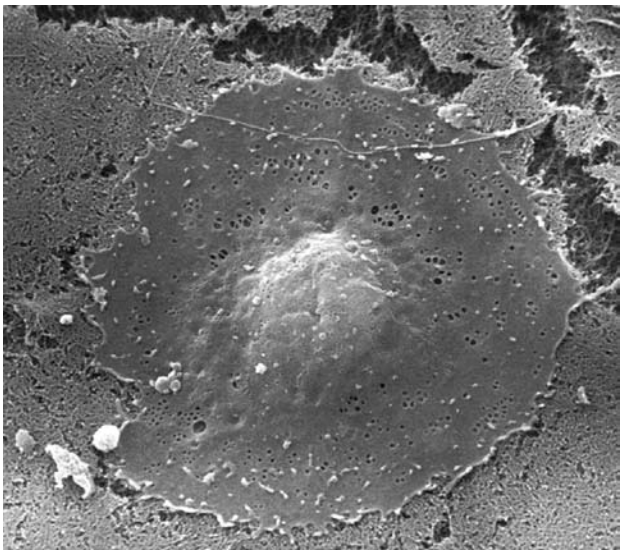


Figure 3 Scanning electron microscopy (SEM) image of a critical-point-dried liver sinusoidal endothelial cell in culture, 2500 ×. After isolation and purification, cells were seeded on a layer of collagen. Besides the centrally located, bulging nucleus, fenestrae can be easily observed in the surrounding thin, flat cytoplasm.

Preparing sections for LM

Follow the instructions given for TEM and make sections of 0.5-1 μm, stain with Toluidine Blue (1% with 2% borate in distilled water), cover sections with Entellan and a 0.17-μm glass coverslip. In case of sufficient contrast, use bright field microscopy, in case of low or insufficient contrast, use phase contrast or interference contrast.

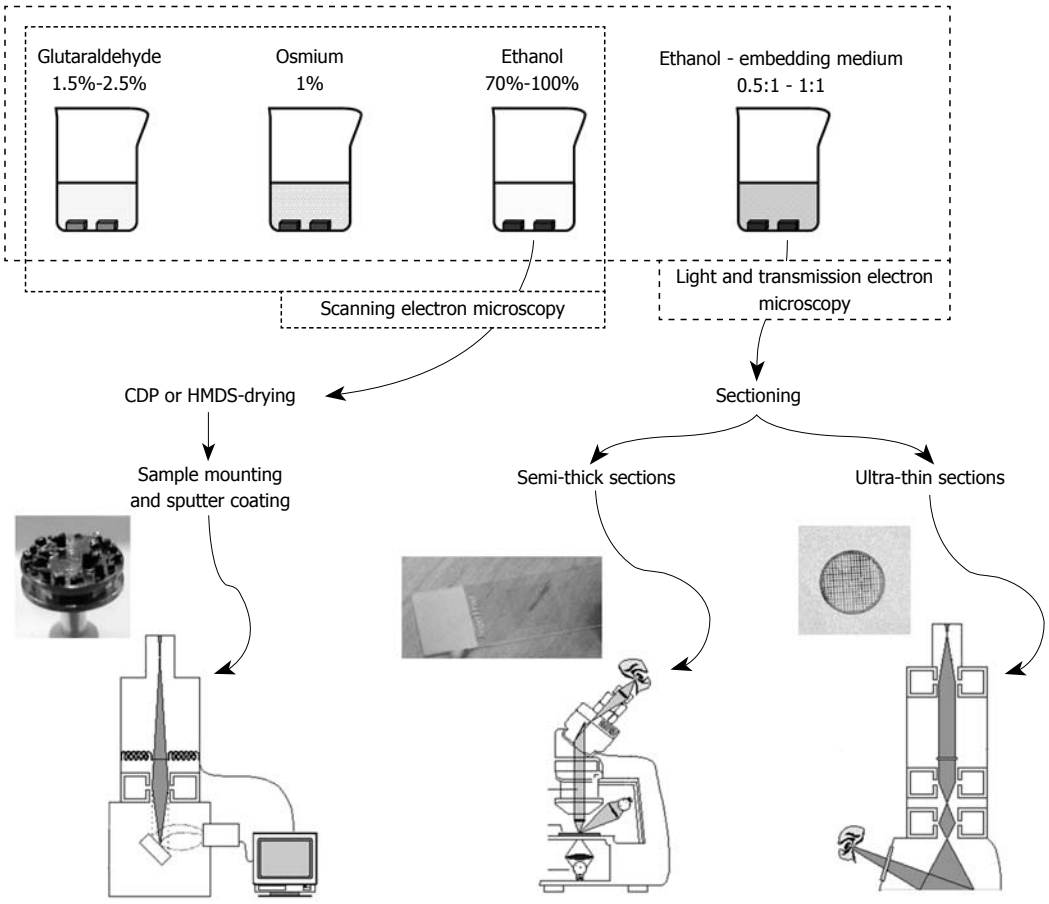


Figure 4 Summary of the different steps in the preparation of liver tissue after aldehyde fixation (top figure) and the subsequent tissue sample preparation steps for the different modes of microscopy (bottom figure). Good laboratory practice should involve all three visualization modes in order to collect fine structural and topological data, together with insights into the interior of the sample. Left: SEM; Middle: Light microscopy (LM); Right: TEM.

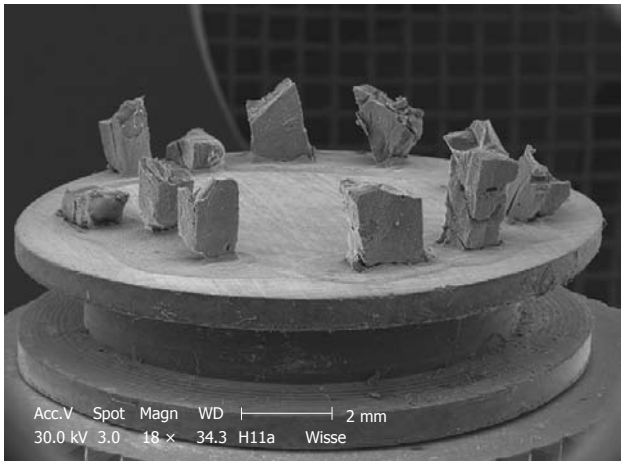


Figure 5 Typical stub of aluminum (diameter 1 cm) bearing 11 pieces of liver tissue of an injection-fixed human liver wedge biopsy observed by SEM at low magnification. Strips of tissue in 100% ethanol are frozen at -196℃, fractured, critical point dried, sputter coated and glued to this carrier. The top surface of the specimens is observed, and imaging is achieved with secondary electrons. This picture is taken by a small CCD camera fitted to the microscope to observe specimens directly.

KEY ASPECTS TO CONSIDER

Anesthesia

When using experimental animals, fixation should be preceded by anesthesia. Undoubtedly, the success of perfusion also depends on the handling of the animals. Animals should be minimally stressed before and during the operation. Therefore, it is recommended to have the animals about 2 h before the surgical procedure within the operating room to accustom them to the environment and reduce the level of stress. Stress hormones affect the physiology and fine structure of organs. Furthermore, animals in discomfort are not only difficult to manipulate, but the stress hormones might cause vasoconstriction and directly narrow or even block some vascular beds, thereby affecting the outcome of the perfusion procedure and also the fine structure of the cells.

Some researchers use ether as an anesthetic. Although this is a rapid procedure, it should be discouraged as it does not provide any pain relief, nor does it have muscle-relaxant properties. Furthermore, ether anesthesia is difficult to control, and often leads to an overdose, typically to be encountered during the initial stages of the operation, resulting in body temperature drop, shock and even accidental death of the animal. Animals that are in distress and anesthetized with ether are difficult to handle and operate on and chances for a successful liver perfusion are low. As outlined in the protocol section, agents such as pentobarbital and chloral hydrate, preferably combined with pain inhibitors, are the first-choice anesthetics. The

period of anesthesia should be as short as possible[22]. Anesthesia can be checked by gently touching the cornea of the animal, followed by gently squeezing the paw pads. When no reaction occurs, the animal is ready for operation. When laparotomy is performed, hypovolemia should be prevented and veins and arteries should be well perfused and red[22].

Preperfusion

The need for preperfusion with buffered physiological saline is still being discussed. The argument used in its favor is to wash plasma and blood cells away in order to avoid coagulation, precipitation of blood constituents and aggregation of blood cells by glutaraldehyde. Such precipitates could impair the flow of fixative through the liver. Theoretically this is correct, but experience has taught us that plasma constituents and blood cells do not have enough time to react and precipitate when glutaraldehyde is flushed through the liver straightaway. It is possible to flush the liver, e.g. of a rat, within 15-30 s without any notable obstruction. After 30 s, the way should be free to continue perfusion of the fixative. The point is that fixative and biological material need time to react, and perfusion might be successfully established before precipitation, coagulation and cellular aggregation occurs. A compromise might be to fill the tubes used for perfusion with buffered physiological saline, which results in a small "wave" of saline preceding the glutaraldehyde, the two being sequentially distributed through the entire microcirculation of the liver[22].

When preperfusion is still preferred, not more than 30 s preperfusion with buffered physiological saline at a physiological flow rate is necessary to free the liver of plasma and red blood cells. From this moment onwards, glutaraldehyde fixation can be started. The success of perfusion correlates with disappearance of red blood cells from the liver and can be followed by the change in color of the organ (dark red brown to yellow-brown). The presence of blood can also be monitored by observing the effluent. The success of fixation correlates with the change in consistency of the organ (from soft and pliable to rigid/stiff like a hard-boiled egg). Successful perfusion fixation shows the start of these phenomena within 20 to 30 s.

Air bubbles might form within the flexible tubing that connects the perfusion needle with the vessels that contain the perfusion fluids. Bubbles appear often when improperly cleaned tubing is used, or are caused by long waiting times or temperature differences between the different fluids. Air bubbles are disastrous in liver perfusion and act as blockers of the liver microcirculation. They are a major source of perfusion failure[5]. It is therefore recommended to check the tubing regularly. Air bubble traps are commercially available and are being used in clinical settings. They certainly are advised when using larger animals (e.g. rabbits, dogs or mini pigs) when high-volume perfusion is required.

As soon as perfusion is started, it is mandatory to open the vascular system by incising the vena cava in order to avoid the buildup of pressure. Enhanced pressure damages endothelial cells as well as parenchymal cells[28].

Formaldehyde has found no application in perfusion fixation of the liver. Glutaraldehyde has better stability in solution and has two reactive groups per molecule, therefore, it cross-links more easily, has lower osmotic pressure and gives better results[29]. Nevertheless, it might be useful to preperfuse the liver with formaldehyde. Since formaldehyde has a much slower reaction time than glutaraldehyde[29], it could be possible to preperfuse the liver with 3%-4% buffered (and freshly prepared) formaldehyde. Care should be taken for the high osmotic pressure of formaldehyde solutions. Formaldehyde preperfusion would allow flushing of blood and its constituents from the tissue (within 1 min) and starts mild fixation at the same time. Glutaraldehyde perfusion can be started directly thereafter and makes clear the way to do the real job of fixation in the absence of plasma and blood cells.

The choice of a roller pump *vs* gravity perfusion is a matter of personal preference. The roller pump releases moderate artery-like pulses of fluid (depending on the tube diameter), whereas gravity flow is constant and results in constant flow or pressure. The choice is considered not to be decisive for perfusion quality.

Since the liver circulation can be characterized as a high flow/low pressure system, discussions about perfusion pressure during fixation have limited value. It is believed that flow rates within wide limits do not enhance perfusion pressure, since the open system and sponge-like structure of this typical microcirculation do not allow pressure to build up. This situation changes when the fixative precipitates blood proteins, aggregates blood cells and introduces coagulation. When attention is paid to this condition, physiological flow rates (the rule of thumb: weight of liver in grams = milliliters of fluid per minute) should give good results.

Choice of the perfusion needle

Experience over the years has taught us that the outcome of fixation by perfusion also depends on the choice of the needle. The diameter of the needle, i.e. the gauge (G), determines the maximum flow to be delivered. This gauge value, together with the liver blood flow and pressure for a particular type of species, are important factors to consider. 18 G needles, for example, with a typical diameter of 1.2 mm, have a maximum perfusion flow by 12 cmH_2O gravity of 80 mL/min. This is evidently too low when livers of rabbits (177 mL/min) or dogs (303 mL/min) are concerned[30].

18-23 G needles (diameter 1.27-0.64 mm) are ideal for liver perfusion in animals such as rats and mice (Figure 1D1); whereas the 12 G (diameter 2.77 mm) needle type is recommended for larger species such as rabbits and minipigs (Figure 1D3). One can benefit from studying a catalogue of the needle catheter-type of needles that are on the market. A needle catheter (Figure 1D2) comprises a metallic needle-guide that consists of a cannula with an end grip - the catheter - that covers the needle-guide when it is retracted after cannulation. Hence, after the needle

catheter (inlet) is inserted into the portal vein and secured by ligatures, it is possible to insert the plastic tube (outlet) further into the portal vein, without rupture of the vessel. An additional benefit is that the atraumatic outlet of the catheter has a round shape, which results in more efficient perfusion when compared to the pointed-end needles. Accidental puncture of the wall of the portal vein at the start of perfusion is a major source of perfusion failure. In case of perfusion failure, an emergency solution is offered by the technique of injection fixation. In that case, the steps of protocol 2 should be followed. This might save losing an animal from the experimental setup.

A point of interest in choosing the right diameter of needle lies also in the effect of "jet streaming" that is caused by a too-small needle orifice. With an easy, moderate flow, the fluids probably distribute evenly through the branches of the portal vein, whereas a jet-stream might, simply because of its strength and direction, blow the fluids into one branch of the portal vein, thus causing uneven distribution and pressure of fixing fluids in the different liver lobes.

When injecting glutaraldehyde into a wedge biopsy, one should take care to introduce a fluid-filled needle into the tissue. When the needle cuts the tissue as it does in the case of a needle biopsy, the sampled tissue comes out at the moment the operator starts injecting the fixative. Instead, if the operator fills the needle with fluid and cuts through the tissue without allowing the tissue to enter the lumen of the needle, this effect can be avoided. Moreover, when the fluid-filled needle is retracted slightly after reaching the maximum track for injection (evidently before starting perfusion), a space will be created within the tissue, from where fluids will distribute easily in all directions as soon as the injection of the fixative solution starts.

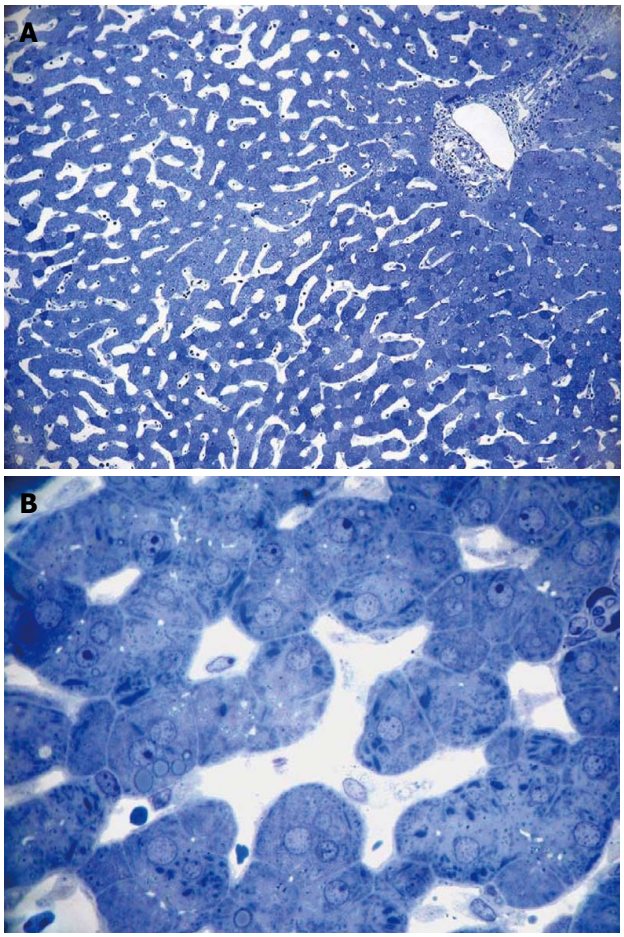


Figure 6 Light micrograph of a wedge biopsy of human liver, injected with glutaraldehyde, plastic section stained with Toluidine Blue. A: Original magnification 10 × objective lens. Note that, as a result of successful perfusion, sinusoids are open, and only a few red blood cells are present. A portal tract is present in the upper right corner; B: Original magnification 63 × oil-immersion lens. Details such as giant mitochondria are visible in the parenchymal cells, amongst others such as small mitochondria, lipid inclusions and bile canaliculi. Sinusoids are patent and show the presence of sinusoidal cells.

RESULTS OF THE DIFFERENT METHODS OF FIXATION

When LM sections, SEM preparations and ultrathin sections of liver tissue or cell cultures are studied, these three approaches provide information to the investigator, each in its own way.

Toluidine-Blue-stained 1-μm sections provide information at magnifications from 5 × to 1000 × over wide areas as compared to TEM sections (Figure 6A and B). These sections already show the criteria for good or bad fixation (see discussion), such as open sinusoids (Figure 7A and B), absence of blood cells, equal density of similar cells, and open bile canaliculi (particularly in the periportal area). Recognition of typical histological details such as the central vein, bile ducts and portal vein with its accessory structures and many cells, such as parenchymal, sinusoidal and visiting cells, together with conditions such as steatosis (Figure 8A and B), fibrosis and inflammation, can be observed at an estimated system resolution of about 0.2 μm. Crucial details such as endothelial fenestrae (measuring 140 nm in mice and rats, and 105 nm in humans and some rabbit strains) (Figures 9-12), although present, therefor escape observation by LM. Perfusion fixation and plastic embedding also add details to an LM study. In the case of immersion fixation, paraffin embedding and hematoxylin and eosin staining, observations are mainly restricted to the size, shape and position of entire cells and their nuclei. In the case of perfusion fixation and plastic embedding, intracellular details can be observed in the LM in greater detail, with the knowledge that the same structures of the same preparation can also be seen in maximal detail by TEM. For example, the typical giant mitochondria that occur in the human liver parenchymal cells (Figures 13 and 14) can easily be observed in plastic sections by LM, and can be seen in greater detail by TEM of the same specimen. Therefore, the Toluidine-Blue-stained plastic section is a useful histological introduction to further detailed study by TEM.

Perfusion-fixed total organs or injection-perfused wedge biopsies also make ideal preparations for SEM study. When tissue strips of 1 mm × 1 mm × 5 mm in 100% ethanol are frozen and fractured (before CPD) at the temperature of liquid nitrogen, the fracture surface is

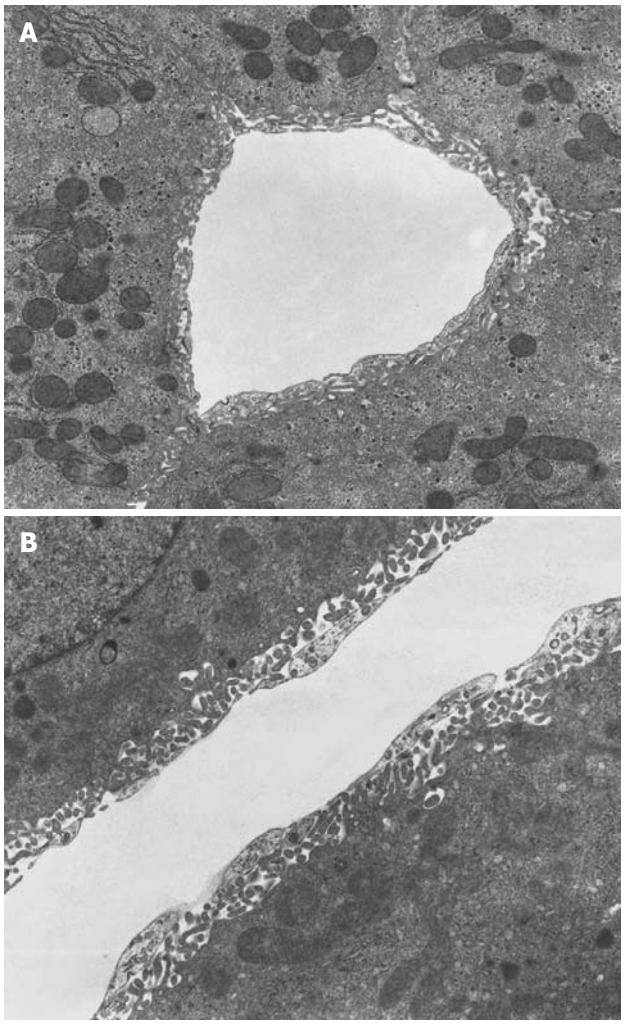


Figure 7 TEM micrograph of a transversely cut sinusoid of rabbit liver (A) and longitudinally cut sinusoid of mouse liver (B), fixed by perfusion through the portal vein. A: The Space of Disse shows the presence of microvilli extending from the parenchymal cells. Within the cytoplasm of the parenchymal cells, mitochondria, rough endoplasmic reticulum and glycogen are recognizable. Original magnification 6600 ×; B: Underneath the thin layer of fenestrated endothelium, bordering the sinusoidal lumen, the Space of Disse shows the presence of microvilli extending from the parenchymal cell surface. Within the cytoplasm of the parenchymal cells, mitochondria are recognizable. Original magnification 8900 ×.

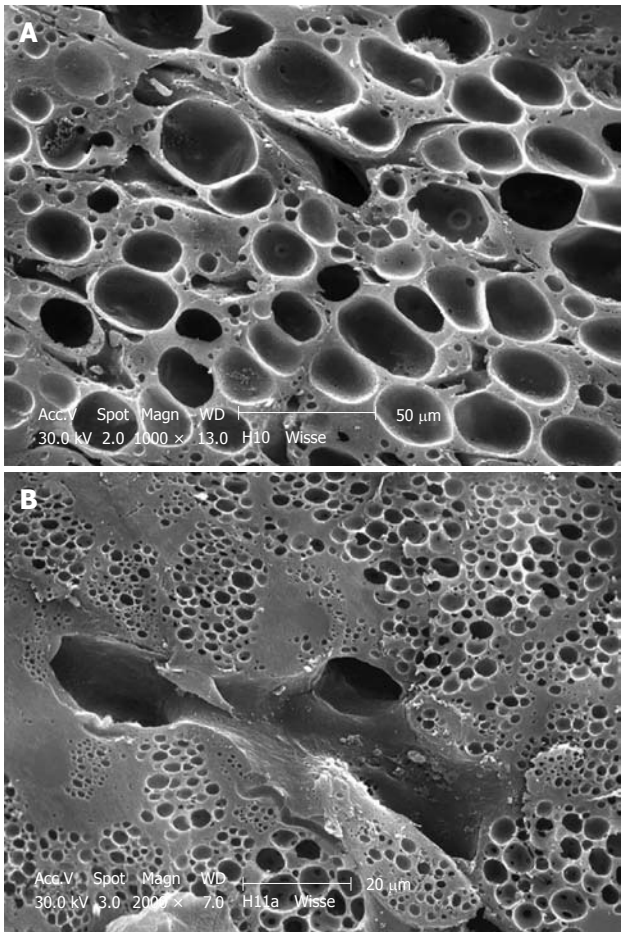


Figure 8 SEM micrograph of an injection-fixed human wedge biopsy showing macrovesicular steatosis (A) and microvesicular steatosis (B). A: Fat droplets are of such dimensions that one appears to fill the cytoplasm of an entire parenchymal cell; B: Fat droplets are smaller than those in Figure 8A and are spread within the cytoplasm of parenchymal cells.

more or less flat and easily correlates with a 2D histological section. Unfortunately, intracellular details are rare, but some cell surface details are abundant. SEM offers details about the endothelial lining of the sinusoids, easily shows the presence of fenestrae (Figures 11 and 12), reveals the presence of endothelial gaps (Figures 15 and 16), shows some details about the space of Disse, and shows the central and portal veins and the bile duct with its typical epithelial cells with one cilium each (Figure 17). Alternatively, fracturing the tissue in the already dry state after CPD (Figure 17) reveals the lateral cell membranes of parenchymal cells together with the 3D network of bile canaliculi, strictly separated from the network of sinusoids. SEM preparations are very useful in judging conditions such as sinusoidal dilatation (Figure 18) or sinusoidal obstruction syndrome[31]. Sinusoidal dilatation occurs in regions apparently different form normal, where the average sinusoidal diameter is measured as 4-6 μm by SEM. In SEM preparations, the dimensions of many structures are reduced by an average 30% shrinkage as a result of the drying procedure[23,24]. Therefore, the use of SEM preparations to measure or judge the size of any detail in the preparation should be avoided.

TEM of perfusion or injection fixed liver tissue reveals the fine structure of all the different cell types in the tissue. It is assumed that the histological topography and the shape of the cells are optimally preserved due to hardening of the intact tissue by glutaraldehyde perfusion. This initial hardening process needs only a few minutes for the entire organ. Important to note is the fact that this condition can be obtained without touching or deforming any part of the organ or tissue. It is assumed that the fluids themselves do not cause distortion as reported for immersion fixation[19].

Inherent to the observation with TEM, abundant details can be observed in all different cells and organelles at magnifications in the range of 50-1 000 000 ×, with a resolution in the order of about 5 nm. A low magnification survey of sections at 50-600 × (by using a different

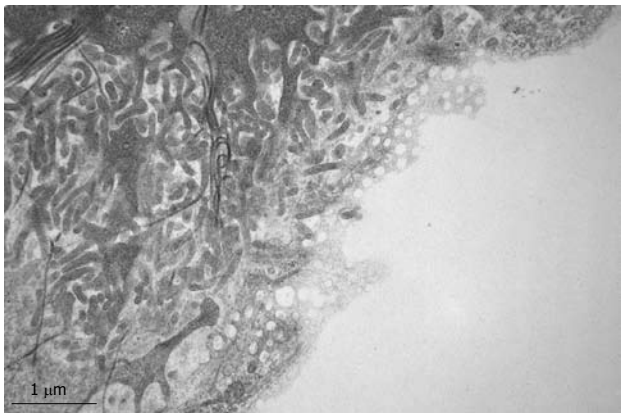


Figure 9 TEM micrograph of the tangentially cut endothelium of a human liver sinusoid, fixed by injection of glutaraldehyde fixative into a wedge biopsy. Underneath the thin layer of fenestrated endothelium, the Space of Disse shows the presence of microvilli, thin fibers of reticulin, and processes of fat-storing cells. Fenestrae in such preparations have an average diameter of about 105 nm. Original magnification 19 000 ×.

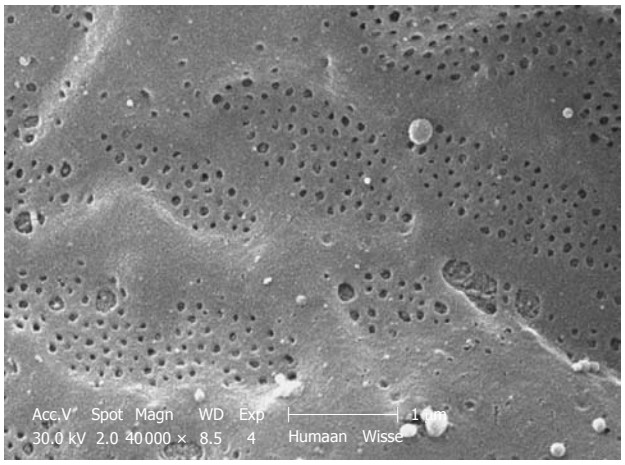


Figure 11 SEM micrograph of the fenestrated endothelial lining of a human injection-fixed wedge biopsy. Note the grouping of fenestrae in sieve plates. Compare this figure with Figure 12, which shows an SEM micrograph of a mouse sinusoid at the same magnification. Fenestrae are apparently sensitive to fixation and are lost during immersion fixation. Their presence can therefore be used as a criterion of good fixation.

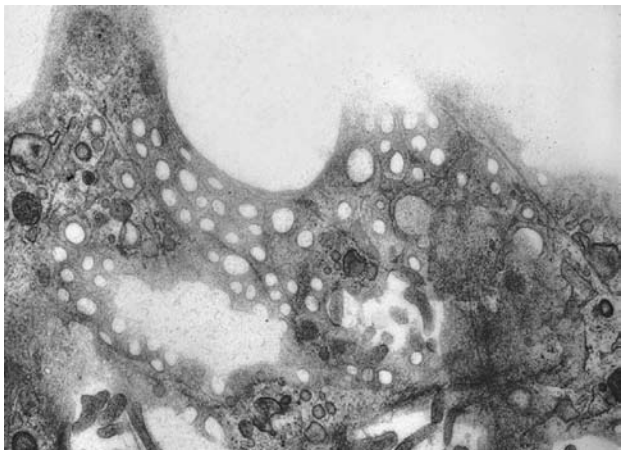


Figure 10 TEM micrograph of tangentially cut sinusoidal endothelium of a rabbit liver, fixed by perfusion through the portal vein. The fenestrae are grouped in sieve plates, but are intermingled with elements of the cytoskeleton, i.e. microtubules and microfilaments. Within the electron-dense sieve plate cytoplasm, saccular interconnecting cisternae are seen. These structures are fixation dependent and are only found in rabbit liver. Original magnification 21 000 ×.

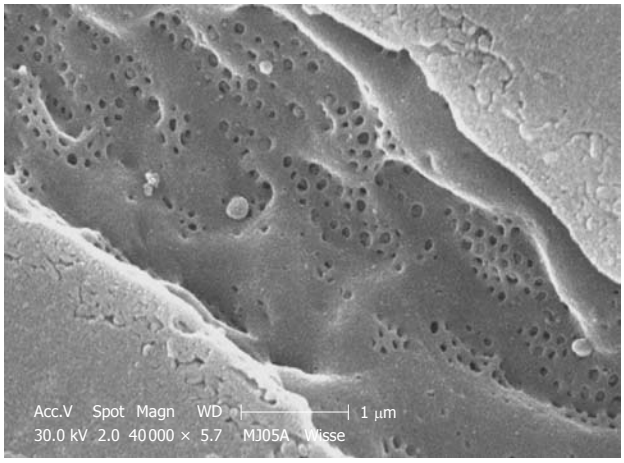


Figure 12 SEM micrograph of the fenestrated endothelial lining of a mouse portal-vein-perfused liver. Compare this figure with Figure 11, taken under exactly the same conditions. Comparison with TEM of plastic sections shows that mouse (and rat) fenestrae are bigger (140 nm) than human (and rabbit) fenestrae (105 nm). Measurements on this type of SEM preparation should be avoided, because the drying procedure results in about 30% shrinkage and incorrect measurement of all components in the tissue.

combination of TEM lenses) (Figure 19), provides information about the quality and content of the sections, and allows the preselection of interesting areas in the preparation. This low magnification TEM is also recommended as a bridge to the study of Toluidine-Blue-stained sections for LM. TEM observations also reveal the difference between sinusoids and capillaries (Figures 20 and 21). Perfusion fixation also reveals extraordinary structures such as pored domes[32] or defenestration centers (Figure 22)[33]. Higher magnifications provide supramolecular information about the cytoskeleton, glycogen rosettes, ribosomes, mitochondrial cristae, chromatin fine structure, nucleolar composition, unit membrane structure, Golgi apparatus, the presence of iron storage proteins, and lysosomal contents.

Results obtained with cell cultures follow the general pattern described for the three kinds of specimen already discussed. In the case of cultured cells, fixation at the cellular level is quite easy. A big difference is that fluids have direct access to the cells. Therefore, precautions should be taken to avoid over-fixation and extraction, which can result in damaged membranes and extracted cells.

GENERAL DISCUSSION

A detailed literature survey has revealed that there are three commonly used fixation methods for EM studies of liver tissue. For a review see Hayat[21]. Since Fahimi[5] and Wisse[6] have demonstrated that perfusion fixation, rather than immersion fixation, is essential in preserving the fine structure and architecture of the liver, perfusion-fixation has been the gold standard ever since, especially

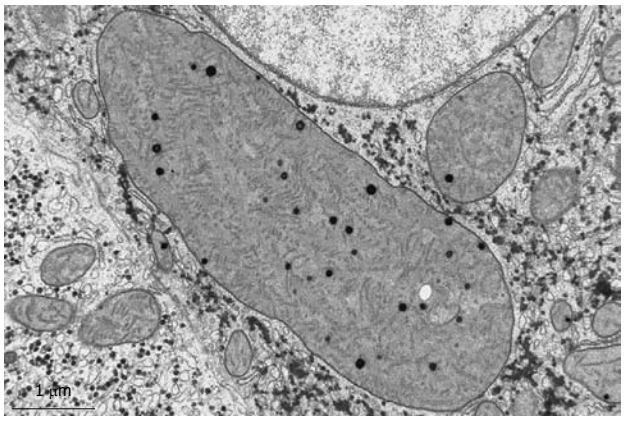


Figure 13 Typical structures that are often encountered in human liver parenchymal cells are the giant mitochondria. This wedge biopsy was fixed by injection, but during the osmium postfixation, ferrocyanide has been added. This compound enhances the contrast of membranes, including the cristae. Giant mitochondria also contain electron-dense granules, as normally seen in small mitochondria (compare Figure 14). Giant mitochondria can also be recognized in plastic sections observed by LM (see Figure 6B). Magnification 19 000 ×.

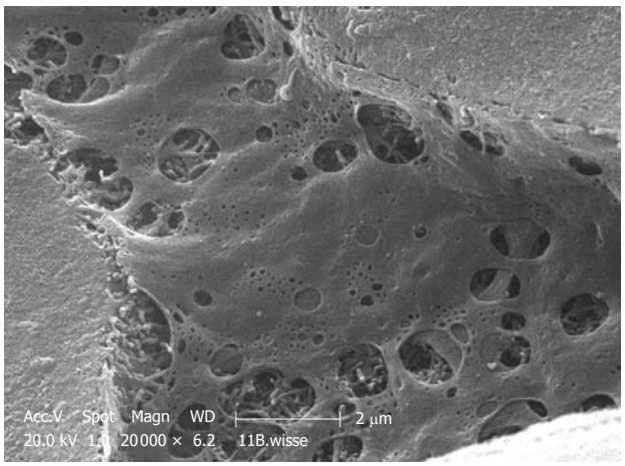


Figure 15 A major problem in EM preparations of liver tissue is the occurrence of gaps in the endothelial lining. It is assumed that they appear during fixation due to instability of the cytoskeleton, which allows the fusion of fenestrae or the *de novo* formation of gaps. This SEM picture is from a rabbit liver fixed by portal perfusion.

for the study of the sinusoids and sinusoidal cells in experimental animals[6].

Irrespective of the numerous methods available to prepare liver tissue for EM studies, perfusion-fixation *via* the portal vein is decidedly superior and more reproducible compared to other methods of fixation, in particular immersion fixation. Injection of the fixative into wedge biopsies, however, comes close and when Toluidine-Blue-stained LM sections are used to select tissue for the quality of fixation, this type of fixation can replace that through the portal vein. Perfusion-fixation *via* the portal vein and immediate arrest of the liver circulation (including the hepatic artery), meets the conditions of instantaneous and simultaneous fixation of all cells in the organ and restricts gradients of fixation to the cellular level. Fahimi[5] and Wisse *et al*[22] have reported

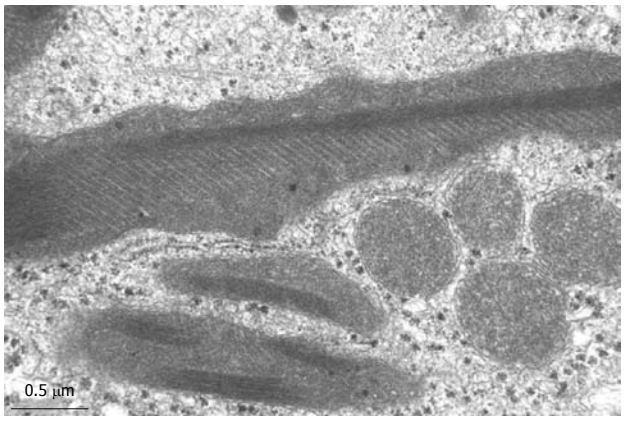


Figure 14 Giant mitochondria in a wedge biopsy fixed by injection. No ferrocyanide has been added during the osmium postfixation, therefore, membrane contrast is low. Within the giant mitochondria, the occurrence of typical crystals can be observed, together with a highly rhythmical pattern of the cristae. Magnification 34 000 ×.

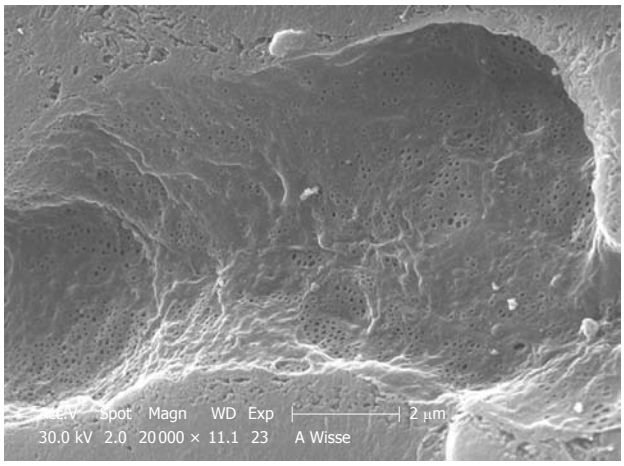


Figure 16 SEM micrograph of another portal-vein-perfused rabbit liver, which shows a complete intact sinusoid, and apparently no gaps have been formed. Fenestrae occur in sieve plates (groups), the space of Disse seems to be filled by microvilli of the parenchymal cells.

the success of portal vein perfusion, followed by retrograde perfusion with fixative through the hepatic vein, which results in more evenly fixed tissue. By staining with Schiff's reagent, we could demonstrate that within 5-7 min, liver tissue stains equally well after such "push-pull" fixation[22], which indicates that saturation of the tissue with glutaraldehyde is obtained over this short time interval. Perfusion through the aorta[28] or any other indirect route may be successful, but seems unnecessary when the portal vein is available. We can summarize the advantages of perfusion fixation as follows: (1) instantaneous and simultaneous fixation of all cells in the organ; (2) avoidance of hypoxia and autolysis; (3) fewer problems resulting from diffusion and exhaustion of the fixative during immersion; (4) use of lower concentrations of fixing chemicals is possible; (5) glutaraldehyde perfusion hardens the tissue, which allows easier cutting; (6) less mechanical damage, deformation or distortion; (7) empty vessels and sinusoids allow better penetration of

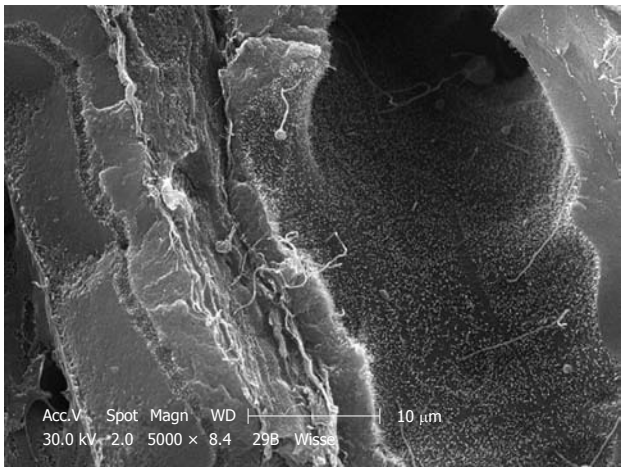


Figure 17 SEM micrograph of rabbit liver, fractured after CPD. In such preparations, the fracture plane separates the intact lateral cell membranes of parenchymal cells, which exposes the bile canaliculi, which are strictly separated from the sinusoids (to the extreme left). The right hand side of the picture shows a bile duct. The luminal surface of the bile duct epithelial cells is covered with numerous small microvilli and single cilia protruding into the lumen of the bile duct.

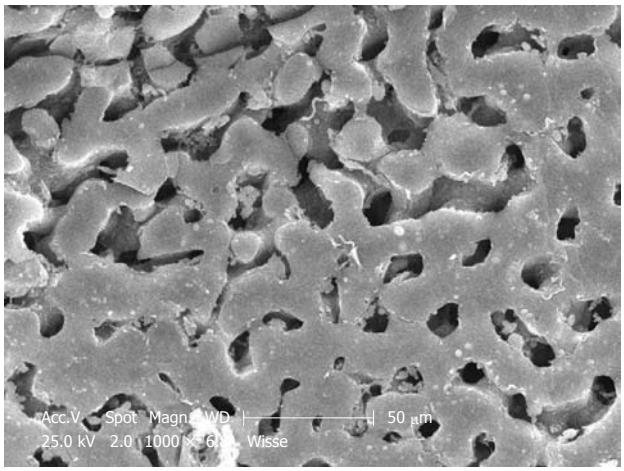


Figure 18 SEM micrograph of a human injection-fixed wedge biopsy that shows two populations of sinusoids, i.e. normal size (lower half of the picture), and those in the upper half of the picture show sinusoidal dilatation.

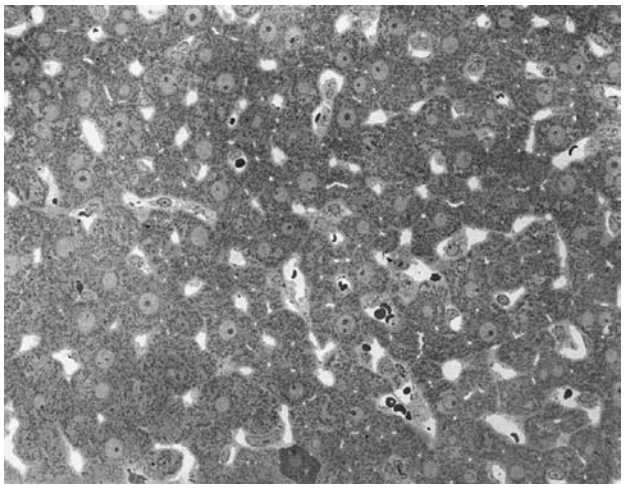


Figure 19 TEM image after perfusion fixation of rat liver through the portal vein, which results in an image of parenchymal cells of equal density (with one exception in the low middle part), open sinusoids and open bile capillaries. Original magnification 720 ×.

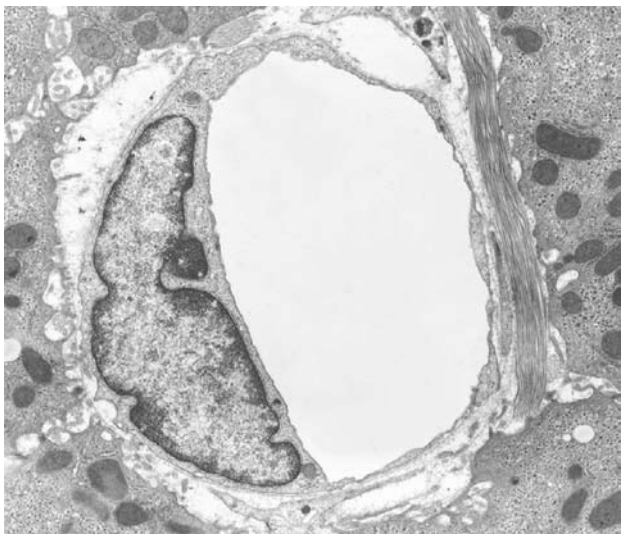


Figure 20 TEM micrograph of a capillary found in diseased human liver, fixed by injection of fixative into a wedge biopsy. Capillary endothelial cells are different from normal sinusoidal endothelial cells; their vacuolar apparatus (pinocytotic vesicles, endosomes, lysosomes, Golgi apparatus) is not developed. The thin cytoplasm contains fenestrae and the capillary is surrounded by a thin, continuous basal lamina that is not present in normal sinusoids. Magnification 6600 ×.

washing fluids, osmium and ethanol; (8) exact timing and shorter fixation times are possible; (9) more and better preservation of histological relationships and fine structural details; (10) better preservation of cytochemical reactive sites; (11) fewer artifacts; (12) perfusion can be extended to a program of reagents, fixatives and buffers without further manipulation; (13) less accessible organs and tissues may be fixed before excision; and (14) easy to directly judge good perfusion and fixation by change in color and hardening of the organ.

An additional advantage of the method is that perfusion-fixed liver tissue is firm enough to be cut into finer pieces without deformation, supposedly leaving cells in their right histological arrangement. Hand cutting by a sharp, thin razor blade allows the cutting of glutaraldehyde fixed tissue into tissue blocks of 1 mm × 1 mm × 1 mm (for TEM) or slices (1 mm × 10 mm × 10 mm) for flat embedding and large sections for LM, and strips of 1 mm × 1 mm × 5 mm for further preparation for SEM. It is assumed that the open vessels and sinusoids allow the penetration of osmium and other fluids in a much better way as compared to immersion fixation, for which a wall of fixed components at the outer rim of the tissue stands in the way of and will still react with the penetrating fluid.

Researchers may choose from two perfusion methods that both result in excellent preservation of hepatic tissue. One uses gravity to perfuse the vascular bed at 12 cm water pressure, a value that corresponds to the physiological portal pressure (Figure 1A); whereas the other method actively pumps solutions through the portal vein with the

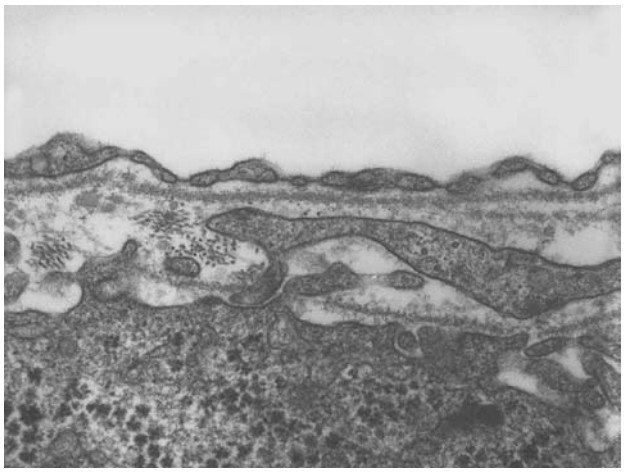


Figure 21 TEM micrograph of a capillary found in diseased human liver, fixed by injection of fixative into a wedge biopsy. The thin endothelial lining contains fenestrae, closed by a diaphragm. Underneath the endothelium, one can observe a continuous basal lamina. Fenestrae in this type of capillary are about 60 nm in diameter. It is supposed that transport from this capillary to the tissue is hampered by the structures mentioned. Magnification 28 500 ×.

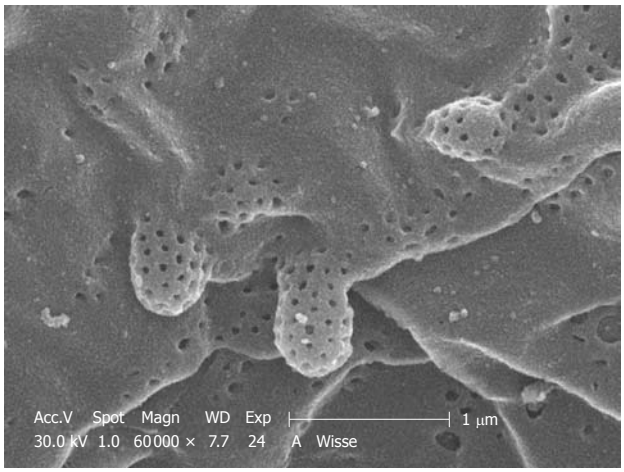


Figure 22 SEM micrograph of a portal-vein-perfused rabbit liver. Due to the quality of fixation, fenestrae are preserved, including a surprising detail, named pored domes[32], which are probably the *in vivo* equivalent of the defenestration center observed in endothelial cell cultures[33]. This structure was not seen in human, mouse, rat or pig liver.

aid of a low-speed peristaltic pump. In both cases the typical rate of 1 mL/min per g liver weight (Figure 1B) should be achieved.

Perfusion fixation seems to be difficult when a human needle biopsy is to be investigated by EM. Attempts have been made by Bioulac-Sage *et al*[14] and Balabaud *et al*[8] to flush fluids through the tissue of a liver needle biopsy arrested at one end of a narrowing Pasteur pipette (holding the tissue) with a slightly elevated pressure, while aspirating fluids at the other end with slightly negative pressure. Muto *et al*[10], Vonnahme[12] and De Wilde *et al*[15] have obtained good fixation results on human or rat liver needle biopsies by injecting glutaraldehyde with tiny glass or metal needles at intervals into the cylindrical tissue. De Wilde *et al*[15] have used low-melting-point agar to arrest and handle the tissue. Surprisingly, and in spite of good results, this technique has not found its way into clinical practice, and it is not mentioned in the needle biopsy review of Rockey *et al*[3].

It is evident that immersion-fixed needle biopsies have provided a wealth of information about the fine structure and deviations of parenchymal cells. David *et al*[34] have demonstrated by morphometric methods that there are only slight differences between parenchymal cells from immersion- or perfusion-fixed tissue. It is recommended to limit the study of immersion-fixed needle biopsies to the outer and middle zone of cells that are well fixed. They can easily be found when material is preselected by Toluidine-Blue-stained LM sections. Apparently, the structure of sinusoids and sinusoidal cells, as damaged as they are after immersion fixation, has not until now played a significant role in clinical practice.

With regard to artifacts, we remember a citation from one of the founding fathers of EM, George Palade, who said that one of the major difficulties in EM is to distinguish between genuine structures and artifacts. Some even say that EM is "an art of artifacts". It takes indeed a lot of experience and insight to distinguish between real structures and artifacts. Alternative methods are scarce, but sometimes it helps to compare results of chemical fixation (as summarized in this review) with alternative techniques such as freeze fracture of fresh tissue[6]. In equilibrium with the fact that artifacts are not easy to define and to prove, it is also difficult to indicate the true nature of real structures and their function. Artifacts in a specimen can be caused during one or more of the many steps of sample preparation, and can be caused by anesthesia, osmotic pressure, quality of the reagents, inadequate operation, pH, temperature, postmortem changes or autolysis, slow or incomplete fixation, cutting of the tissue, postfixation, dehydration, distortion, embedding, sectioning, contrast staining, microscope conditions, (digital) photography, image storage, and handling. Examples of possible artifacts are the occurrence of endothelial gaps, shrunken cells, light cells next to dark cells (Figure 23), widening of the Space of Disse, changes in cell shape, changes in the rounded shape of the nucleus of the parenchymal cells, condensation of chromatin[19], relocation or disappearance of subcellular components (such as endothelial fenestrae[6]), swollen mitochondria, collapsing and blebbing (Figure 24) of cell membranes and vesicle formation in intercellular spaces.

With regard to the occurrence of gaps larger than fenestrae in the endothelial lining (Figure 15) a lot of discussion has taken place[22,23]. Indirect proof of their artifactual nature is given by the fact that, in intact animals, endothelial fenestrae have an apparent sieving effect on, for example, adenoviral vectors of 93 nm used for gene transfer[35,36]. The appearance of large gaps in the sinusoidal lining can result from osmolarity problems, from destabilization of the cytoskeleton or from the use of a high perfusion pressure during the washing and subsequent glutaraldehyde fixation during the perfusion-fixation procedures[28,34].

Other subtle artifacts are caused by a process often

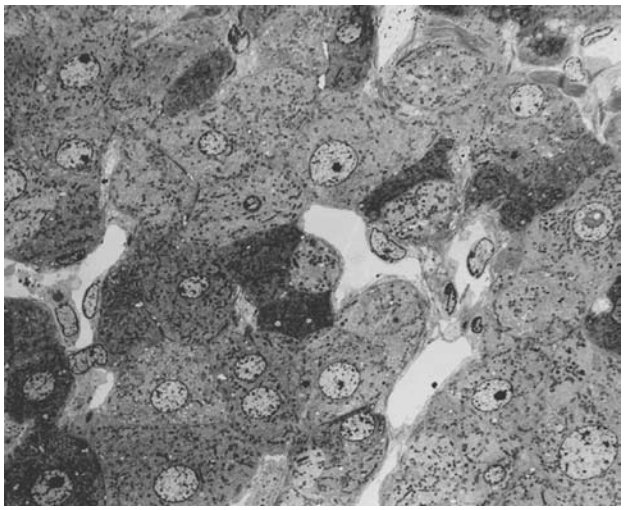


Figure 23 TEM micrograph of an injection-fixed human liver wedge biopsy at 700 × magnification, which shows the presence of dark and light cells next to each other. It is assumed that the differences in density reflect differences in fixation, which often occur in bad fixation.

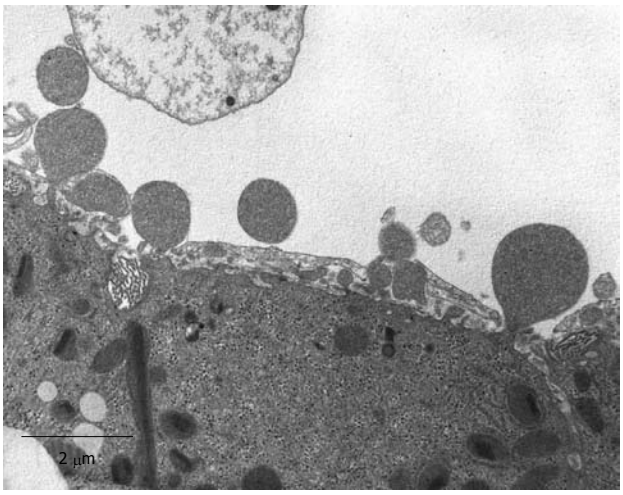


Figure 24 TEM micrograph of an injection-fixed human liver wedge biopsy, which shows the presence of blebbing, which occurs when cells survive in bad conditions. Incomplete fixation is thought to be the cause. Blebs are either swollen microvilli or originate from the peripheral cytoplasm of parenchymal cells. Original magnification 7900 ×.

referred to as over-fixation or "weekend fixation". People sometimes store their precious samples for hours, days or even weeks in glutaraldehyde fixative. Typical for those preparations is that they are rich in free vesicles that seem to form from loose phospholipids derived from the cell membrane. Conversely, blebbing of living parenchymal cells occurs at low oxygen and glutaraldehyde concentrations (Figure 24). Related to this is the clumping of individual glycogen inclusions, poorly staining cell membranes, and failure to achieve staining of glycogen in parenchymal cells. With regard to the transport of material between laboratories, we have good experience of sending samples in 70% ethanol by fast courier services, which are able to deliver between different continents within 1 or 2 d.

How does one assess the outcome of properly fixed liver tissue? In order to distinguish good quality of fixation of the liver, we recommend the following criteria: (1) sinusoids should be open; (2) plasma and blood cells should be practically absent in sinusoids; (3) similar cell types should show similar images; (4) endothelial fenestrae should be present; (5) bile canaliculi (especially in the periportal area) should be open[6,8,22,23]; and (6) nuclei of parenchymal cells should be round (When nuclei of parenchymal cells are elliptic, the nuclei are compressed in the direction of cutting in the ultramicrotome).

The following criteria can be use to distinguish bad fixation: (1) sinusoids are collapsed or largely destructed and still filled with plasma and blood cells; (2) dark and light cells occur next to each other; (3) different images of similar cells; (4) endothelial fenestrae are absent; (5) bile canaliculi are collapsed; and (6) irregularly shaped nuclei of parenchymal cells.

Fahimi[5] has immersed thin slices of perfusion-fixed liver tissue into distilled water. When they change to a white color, the fixation is insufficient. This is a simple, rapid and objective test for good fixation.

Although this paper focuses mainly on the study of ultrathin liver sections with TEM, SEM is also a powerful tool to assess the quality of perfusion-fixed liver tissue. The abundant presence of red blood cells within sinusoids is an indicator of improper perfusion. Sometimes investigators unwarily apply unrelated or unsuitable perfusion or fixation procedures that are fine-tuned for specific applications, tissues, species or even are not applicable for animal tissue material, such as microbial and plant sample preparation protocols. Animal tissue and their cell components have a typical osmotic pressure of 320 mOsmol. Since the osmolarity of the glutaraldehyde component of the fixative seems to be less important[19,37], the osmolarity of the buffers should match the osmotic values of the tissue. Isotonic solutions that include the osmotic pressure of glutaraldehyde have a swelling effect on cells[37]. The protocols described in this review have contributed to the success of EM studies in several groups. The composition of the fluids applied reports the use of sucrose, which is added to a 0.1 mol/L sodium cacodylate buffer to correct its osmotic value to 320 mOsmol.

In vitro perfusion of whole livers has been used often to study preservation conditions of livers for transplantation, or metabolic and physiological processes. It is important to note that perfusion fixation fits well into such schemes and provides essential information about the condition of those livers at the end of an experiment. In addition, perfusion can also be used to incubate the tissue for cytochemical reactions of enzymes, epitopes, and all other reactive elements within the tissue.

CONCLUSION

In a clinical environment, the immersion-fixed needle biopsy is an important standard. The role of needle biopsies in diagnosis, prognosis and management of patients with liver disease[3] makes the needle biopsy a gold standard,

notwithstanding immersion fixation, paraffin embedding, thick 5-μm sections, and the use of low-magnification LM. Together with immunological techniques, specific staining and the professional training of pathologists, needle biopsies fulfill clinical needs. Pathological diagnosis[38] uses pattern recognition in tissues that reveal conditions such as steatosis, inflammation, hyperplasia and neoplasia. EM seems to be of limited or no use in diagnosis[3], in contrast to its use in research. However, EM still has a potential value in a clinical environment.

However, at the other end of the spectrum, there is a group of scientists that knows that perfusion fixation, plastic embedding and the use of EM provides details about cells and tissues down to the nanometer level. Surprisingly, EM does not seem to succeed in helping pathologists to improve further their diagnosis with data derived at this supramolecular level. One of the key elements in this process is, according to the present authors, the lack of use of perfusion fixation for the study of liver tissue from patients. Indeed, we suppose that applying this type of fixation will provide evidence that might be used to the advantage of patients, for understanding the pathogenesis of certain diseases. This was the motivation for writing this review; to summarize the techniques and bring them to the attention of those involved in the process of using microscopic observations to support diagnosis and prognosis of liver diseases. There seems to be a gap between the use of low-resolution LM observations on immersion-fixed, paraffin-embedded, 5-μm sections and high-resolution microscopy of perfusion-fixed tissue. We hope that this review might help to bridge that gap. Essential to this approach is the recognition of the fact that liver tissue should be fixed at the cellular level with fluids that are as close to physiological and physical conditions as possible.

A good fundamental knowledge of the fine structure and histology of the normal liver is needed because some experimental conditions, pathological processes and severe liver diseases result in outcomes comparable to bad fixation. Although it is difficult to decide between genuine and artifactual structures in normal liver, the decision becomes even more difficult when disease conditions are added to the process.

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REVIEW

Inflammation and immunosuppression in severe acute pancreatitis

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Abstract

Acute pancreatitis (AP) is a common disease, which usually exists in its mild form. However, in a fifth of cases, the disease is severe, with local pancreatic complications or systemic organ dysfunction or both. Because the development of organ failure is the major cause of death in AP, early identification of patients likely to develop organ failure is important. AP is initiated by intracellular activation of pancreatic proenzymes and autodigestion of the pancreas. Destruction of the pancreatic parenchyma first induces an inflammatory reaction locally, but may lead to overwhelming systemic production of inflammatory mediators and early organ failure. Concomitantly, anti-inflammatory cytokines and specific cytokine inhibitors are produced. This anti-inflammatory reaction may overcompensate and inhibit the immune response, rendering the host at risk of systemic infection. At present, there is no specific treatment for AP. Increased understanding of the pathogenesis of systemic inflammation and development of organ dysfunction may provide us with drugs to ameliorate physiological disturbances.

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INTRODUCTION

Acute pancreatitis (AP) is a common disease though its incidence varies considerably in different countries from 5 to more than 100 per 100 000 inhabitants/year[1-5]. AP is a disease with wide clinical variation. Most of the patients suffer from mild disease but in about 20% of the cases the patient develops a severe form of the disease, with a mortality of 7%-15%[1,6,7]. The major cause of death is organ failure which complicates severe AP in 20%-80% of the cases[8,9].

AP has many distinct etiologies, though approximately 80% of all cases are caused by either gallstones or alcohol[10]. The frequency of different etiologies varies markedly in different countries. In Finland, alcohol is the causative factor in about 70% of the cases and the incidence of AP correlates with the rate of alcohol consumption[11].

Prognosis of severe AP has improved mainly because of efficacious conservative treatment in intensive care units. Also the correct timing of surgery is important but is indicated only for infected organized pancreatic necrosis when percutaneous drainage techniques are ineffective[12]. In recent years, many minimally invasive

techniques, for example endoscopic and laparoscopic approaches, have been introduced to avoid the need for open necrosectomy[13]. However, in severe AP the incidence of intra-abdominal hypertension is 60%-80% and in some cases necessitates surgical decompression in the early phase of the disease[14]. The use of antimicrobial drug therapy does not protect against infected necrosis or reduce mortality in severe AP[15]. A factor which may affect the outcome is early endoscopic retrograde cholangiopancreatography (ERCP) in biliary AP with biliary obstruction[16]. Thus, despite extensive research during the recent decades, no mode of treatment has been developed that could dramatically improve the outcome of severe AP.

INFLAMMATORY RESPONSE IS SIMILAR IN AP AND SEPSIS

Previously, AP was considered to be a disease of the pancreas. Nowadays, however, there is strong evidence for systemic effects of the disease. The mechanisms of systemic inflammation resulting in organ failures are much alike in severe AP and in, for example, severe burn injury, multitrauma or sepsis[17] (Figure 1). Thus, a considerable amount of research data gained, for example, in sepsis studies could be applicable also in severe AP.

The initial phase of AP involves triggering events that lead to premature activation of pancreatic proteases as a result of intracellular co-localization of the digestive and lysosomal enzymes. This leads to disruption of the acinar cells[18-20]. Then, activated enzymes escape into the interstitium of the pancreas and cause pancreatic autodigestion[21,22]. Furthermore, pancreatic damage is followed by activation of local inflammatory cells and various inflammatory mediators (cytokines) (Figure 1). Localized inflammation is the body's initial physiologic protective response, which is generally strictly controlled at the site of injury. Loss of the local control results in excessive uncontrolled activation of inflammatory cells and mediators. This response is defined as systemic inflammatory response syndrome[23]. Proinflammatory cytokines, tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) are released *via* portal vein and lymph fluid drainage to the circulation[24]. In addition, the production of cytokines enhances proinflammatory signals. IL-6 stimulates the synthesis of acute phase proteins (i.e. C-reactive protein and procalcitonin) in the liver[25]. Proinflammatory cytokines activate the vascular endothelium in the whole body, which leads to enhanced leakage of the capillary veins and triggers migration of leukocytes into tissues. promoting activation of coagulation cascades. Circulating neutrophils and monocytes become activated, as evidenced by the increased expression of adhesion molecules (e.g. CD11b), and release their proteolytic enzymes and oxygen radicals, which damage vascular endothelial cells and organ parenchymal cells[26]. Enhanced amounts of tissue fluid together with impaired microcirculation lead to lack of oxygen, which results in dysfunction of vital organs and, ultimately, in organ failure[27,28]. While lungs and kidneys have a wide capillary bed, they are especially susceptible to phlogistic mediators inducing end organ injury.

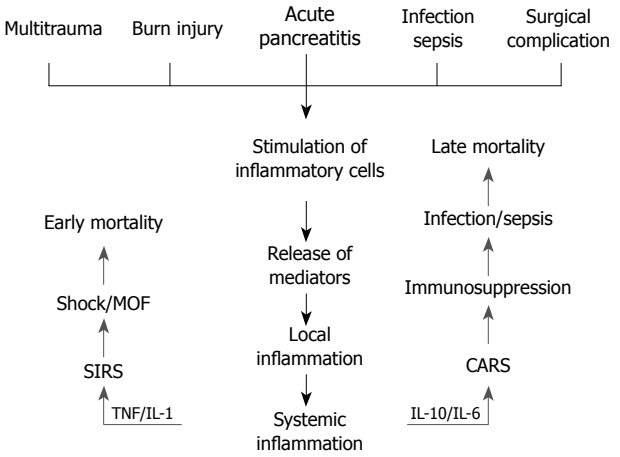


Figure 1 Two-phase hypothesis of development of multiorgan failure. MOF: Multiple organ failure; SIRS: Systemic inflammatory response syndrome; TNF: Tumor necrosis factor; IL: Interleukin; CARS: Compensatory anti-inflammatory response syndrome.

ANTI-INFLAMMATORY RESPONSE

Systemic inflammation in AP is concomitantly associated with rapidly-strengthening compensatory anti-inflammatory response syndrome (CARS)[29,30]. An anti-inflammatory response may be sufficient to control the systemic inflammatory reaction, a sign of good prognosis. However, CARS may be excessive leading to immune deficiency or suppression, which renders the host susceptible to secondary infections. In AP, an increased number of infections has been observed in a later stage of the disease[31], i.e. at the phase of immune suppression.

In immunosuppression, monocytes are characterized by a markedly reduced human leukocyte antigen-DR (HLA-DR) expression, and a profound reduction of their ability to produce proinflammatory cytokines, for example TNF-α[32,33]. Monocytes with low HLA-DR density show impaired antigen presentation capacity[34,35]. IL-10, the most potent anti-inflammatory cytokine, is responsible for the decreased monocyte HLA-DR expression[36]. IL-1 receptor antagonist (IL-1ra) and IL-6 are also important anti-inflammatory cytokines. IL-1ra binds competitively to the IL-1 receptor and blocks IL-1 mediated responses[37]. Previously, IL-6 was regarded as a proinflammatory cytokine. However, some years ago it was shown to act predominantly as an anti-inflammatory cytokine and, for example prevents synthesis of IL-1β and TNF-α[38].

Altered cellular immune function in AP was first reported by García-Sabrido *et al*[39] in 1989 when they showed a correlation between poor outcome and anergy to delayed-type hypersensitivity testing. In addition to proinflammatory mediators[40,41], the IL-10 plasma level has also been shown to be increased in severe AP[42] and predicts development of organ failure in the very early phase of AP[43]. In AP patients, decreased monocyte HLA-DR expression

is associated with development of secondary infections[30]. Decreased monocyte HLA-DR expression has also been shown to predict development of organ failure[44] and a fatal outcome[45]. In AP patients, high plasma levels of IL-10 and IL-6 correlate with decreased monocyte HLA-DR expression[30]. IL-10 and IL-6 can be measured by a chemiluminescent immunoassay that could be available on a 24-h basis at the hospital emergency unit. Flow cytometric measurement of monocyte HLA-DR expression can be used as a routine clinical test and provides results within 30-60 min of sampling. In addition to monocyte functions, there are changes also in lymphocyte count and proportion of T-helper cells that can be measured by flow cytometry in severe AP[46].

DEVELOPMENT OF ORGAN FAILURE WORSENS PROGNOSIS

The classification of AP severity is based on a consensus conference held in Atlanta in 1992[47]. According to the Atlanta classification, AP is classified as severe if systemic and/or local complications (for example pancreatic necrosis, pseudocyst or abscess) are present. Otherwise, AP is classified as mild. Organ failure occurs in half of patients with pancreatic necrosis[48].

The most common organ failure in severe AP is respiratory failure. Also other organs may fail, e.g. renal, hepatic, cardiovascular, digestive, neurologic, coagulation[17] or endocrine or immunologic systems[49]. Failure of different organs has different effects on the prognosis of the disease. Mortality rates in severe AP patients with respiratory, renal, and hepatic failure are 43%, 63%, and 83%, respectively[50]. Lately, the Atlanta classification has been criticized because it does not separate organ failure from local complications[51]. Furthermore, the definition of organ failure in the Atlanta classification is so wide that it includes many patients with a good prognosis. To overcome these shortcomings we have categorized the patients into 3 groups: patients who develop vital organ failure, patients who meet the Atlanta criteria for severe AP but do not develop organ failure, and patients who neither meet the Atlanta criteria nor develop organ failure[41,43,44,52,53]. Indeed, in terms of mortality and morbidity, the organ failure group is the critical one[8,9] regardless of the presence or absence of necrosis of the pancreas[54]. Organ failure often develops early in the course of AP. As many as 37%-76% of AP patients who will develop organ failure may have it already at admission[55,56] or develop it during the first 24 h[44]. Also a difference between transient (< 48 h) and persistent (> 48 h) organ failure has been noted[57,58]. Patients with transient organ failure have an uneventful recovery but persistent organ failure predicts death and local complications.

The mortality of AP is biphasic. Half of the mortality takes place during the first week of the disease and is related to severe multiorgan failure[6]. If the patient survives the first week, he/she may get infectious complications, such as sepsis, with worsening organ dysfunction. These events increase mortality during the second phase of the disease[6]. Knowing the biphasic nature of the mortality is highly relevant to treatment and research of novel treatment modalities in severe AP because the first peak occurs during on-going pro- and/or anti-inflammatory responses whereas the second peak occurs at the phase of immune suppression.

IMMUNE-MODULATION THERAPY

Current treatment of AP remains unspecific and supportive and is mainly targeted to aggressively prevent systemic complications by intensive care. Delayed admission to the intensive care unit results in a 4-fold excess in mortality[59]. Therefore, early identification of patients who develop a severe AP with organ failure is essential for improved prognosis by earlier intervention with appropriate resuscitation in specialized hospitals.

At present, no specific medical therapy for AP exists. Several experimental studies and clinical trials have attempted to discover medical treatment to inhibit the systemic inflammatory reaction and, thus, the development of multiple organ dysfunction. In a rat model of AP, in contrast to expectations, anti-TNF antibody was found to be harmful[60]. However, when given prophylactically anti-TNF antibodies decreased the severity of AP[61]. In experimental AP, block of the cytokine cascade at the level of the IL-1 receptor with a specific antagonist (IL-1ra) decreased pancreatic damage[62]. Furthermore, anti-inflammatory therapy with an IL-10 agonist[63,64] and an anti-IL-8 antibody[65] have shown beneficial effects. In addition, anti-adhesion therapy with anti-intercellular adhesion molecule-1 antibody ameliorates lung injury in mice with severe AP[66]. Platelet-activating factor antagonist lexipafant was promising in the first clinical trials[67,68] but in the final trial the results were disappointing[55]. In the complex network of inflammatory responses, a combination therapy to inhibit several proinflammatory agents could be useful.

In clinical AP, patients are typically admitted to the emergency unit in a fairly late stage of the disease when organ failure is about to develop or may already be present. Then the patient has CARS or probably even immunosuppression, initiation of anti-inflammatory therapies could worsen the prognosis. Thus, the therapeutic window for anti-inflammatory therapies may be too narrow in clinical practice[55]. The problem of early-occurring organ dysfunction in clinical trials is highlighted by the lexipafant study[55], in which 44% of the patients met the criteria of organ failure prior to receiving the first dose of the study drug, an inhibitor of platelet activating factor, or placebo.

In anergic septic patients immunostimulation with interferon-γ has proven to be beneficial[33]. In a clinical study of septic patients, granulocyte macrophage stimulating factor (GM-CSF) treatment was well tolerated and increased monocyte HLA-DR expression and TNF-α production capacity[69]. In severe AP, there is a defect in monocyte function. However, priming of monocytes *in vitro* by interferon-γ and GM-CSF increased HLA-DR expression and restored lipopolysaccharide-induced TNF-α secretion[70]. Yet, it is of note, that immunosup-

pression may be confined to the peripheral blood while the lungs and the other end organs may still be in the pro-inflammatory stage. Therefore, immunostimulatory treatment must be used cautiously and clinicians should have the means to monitor the patients' immunoinflammatory state. Currently, the best method is measurement of monocyte HLA expression by flow cytometry. It would be optimal to depress the pro-inflammatory reaction in the patients at risk of excessive immune suppression so that inappropriate CARS would be prevented. An interesting question is whether the patients in whom a deep immune suppression develops, benefit from antimicrobial prophylaxis, along with immunostimulation.

COAGULATION SYSTEM IS RELATED TO INFLAMMATION

In systemic inflammation, several proinflammatory cytokines activate the coagulation process in the body[71] and activated endothelial cells seem to have an important contributing role in that process. Systemic coagulation activation results in thrombosis in small and middle-sized vessels in many organs, resulting in disseminated intravascular coagulation (DIC), that leads to decreased blood circulation of organs and organ failure. In DIC, consumption of platelets is increased resulting in thrombocytopenia which is a common sign of severe AP[72]. The D-dimer level has been shown to be high in severe AP and serves as a marker of DIC[73]. In experimental severe AP, it was demonstrated that microcirculatory disorders affecting capillary blood flow, capillary permeability and leukocyte endothelial interaction are present not only in the pancreas but also in the colon, liver, and lungs[28].

Protein C is a natural anticoagulant in blood and seems to have an essential role in the regulation of the coagulation cascade in inflammation. Protein C is activated by the thrombin-thrombomodulin complex at the endothelial surface[74]. Activated protein C (APC) inactivates factor V and factor Ⅷ and inhibits thrombin generation. In experimental studies, APC has shown also to have anti-inflammatory effects[75,76]. Septic patients have a decreased protein C level in blood and it correlates with poor prognosis[77]. In meningococcemia patients with DIC, treatment with APC has prevented development of organ failure, and decreased mortality[78]. In patients with severe sepsis, treatment with APC was safe and resulted in decreased mortality[79,80]. In severe AP, protein C pathway defects have been shown to be associated with development of organ failure[81]. In a rat model of severe AP, APC treatment reduced inflammation in the pancreas and lungs and improved survival[82]. These findings encouraged us to perform a placebo-controlled clinical trial of APC in the treatment of severe AP at the Helsinki University Central Hospital.

FUTURE DIRECTIONS

Deeper understanding of the cell biology and physiology of AP is necessary to permit the design of effective interventions concerning the inflammatory response process. Improved methods to accurately identify those patients with severe AP in need of monitoring and treatment in intensive care units are urgently needed. A means to monitor the state of the immune response (monocyte HLA-DR expression) during hospitalization of severe AP patients is important. Whether monitoring signaling pathways of circulating leukocytes, such as nuclear factor κB, signal transducers and activators of transcription and members of the mitogen activated protein kinase family helps us to find the patients at risk for secondary infections and, thus, late organ failure is at present under research[83]. Future treatment strategies will probably focus on combination therapy aimed at suppressing excessive activation of the inflammatory response and preventing concomitantly overcompensating CARS and eventually immunosuppression. Because AP shares many features of sepsis, an urgent question is whether APC provides a means to alleviate severe AP, as it does in patients with life-threatening sepsis. Finally, the analysis of signaling patterns of leukocytes may reveal novel therapeutic targets in severe AP.

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ORIGINAL ARTICLE

CXCR4 antagonist AMD3100 attenuates colonic damage in mice with experimental colitis

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Abstract

AIM: To investigate the effects of the chemokine stromal cell-derived factor-1 (CXCL12) receptor (CXCR4) antagonist AMD3100 on colonic inflammation and epithelial barrier in dextran sulfate sodium (DSS)-induced colitis in mice.

METHODS: Experimental colitis was induced by administration of 5% DSS for 7 d, and assays performed on intestinal segments from the ileocecal valve to the anus. Colonic morphology was examined by hematoxylin and eosin staining. Colonic cytokines were determined by enzyme-linked immunosorbent assay. Myeloperoxidase (MPO) activity (indicator of inflammatory infiltration) was observed spectrophotometrically. Gut permeability was assessed by mucosal-to-serosal clearance of fluorescein isothiocyanate-conjugated dextran 4000 (FD4) in everted gut sacs. The apoptosis of colonic epithelium was assessed by Hoechst-33342 staining. To further elucidate the role of CXCR4 in colonic inflammation, we also investigated the effect of AMD3100 on migration and cytokine production of isolated peripheral blood mononuclear cells (PBMCs).

RESULTS: DSS-induced colitis was characterized by morphologic changes, as well as increased colonic cytokines, inflammatory infiltration, epithelial apoptosis, and intestinal permeability in mice. In AMD3100-treated mice, epithelial destruction, inflammatory infiltration, and submucosal edema were markedly reduced; colonic tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interferon-γ (IFN-γ) levels, as well as MPO activity were significantly decreased. Increased intestinal permeability in DSS-treated mice was significantly reduced by AMD3100. The number of apoptotic cells in colitis mice was markedly increased after DSS administration, and decreased when treated with the CXCR4 antagonist AMD3100. In pre-activated PBMCs, CXCL12 stimulation significantly increased the migration of PBMCs, and was inhibited by AMD3100. Moderately increased TNF-α, IL-6, and IFN-γ from CXCL12-treated PBMCs were also reduced by AMD3100.

CONCLUSION: The CXCR4 antagonist AMD3100 exerts therapeutic effects on experimental colitis by inhibiting colonic inflammation and enhancing epithelial barrier integrity.

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Key words: Ulcerative colitis; Chemokine stromal cell-derived factor-1 receptor; Inflammation; Apoptosis; Intestinal permeability; Epithelial barrier

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INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammatory bowel disease (IBD), clinically characterized by bloody diarrhea, fever, weight loss, and anemia[1,2]. Previous studies demonstrated that pathogenesis of UC involves the interaction of environmental, genetic, microbial, and immune factors, which can initiate an abnormal immune response, and consequent intestinal inflammation[3,4]. Uncontrolled local inflammation disrupts the epithelial lining, resulting in mucosal edema and ulceration, and even crypt abscess in the bowel wall[5]. As in other inflammatory processes, polymorphonuclear leukocytes infiltrating into colonic mucosa are believed to be the main source of cytokine and inflammatory mediators[6]. Chemokines, including growth-regulated oncogene-α and interferon-γ-inducible protein-10, which are expressed on various cells of the intestinal tissues, have been reported to regulate the recruitment of inflammatory cells[7,8]. Block of these chemokines or their receptors significantly attenuates intestinal inflammation and mucosal damage in UC animals, suggesting a pivotal role of chemokines in the pathogenesis of UC[2,8].

The chemokine stromal cell-derived factor-1 (CXCL12) is firstly characterized as a pre-B cell growth stimulating factor and its specific receptor is chemokine stromal cell-derived factor-1 receptor (CXCR4), which also functions as an entry receptor for human immunodeficiency virus[9]. The CXCL12/CXCR4 chemokine axis has an important role in the development of cardiovascular, hematopoietic, and central nervous systems[10,11], and is also involved in several inflammatory diseases such as rheumatoid arthritis, acute lung injury, and sepsis[12-15]. Recent studies demonstrated that CXCL12 and CXCR4 are constitutively expressed on intestinal epithelial cells, lamina propria T cells, and peripheral blood T cells of control patients, and the expression is increased in those of UC patients[16,17]. Block of CXCR4 significantly ameliorates murine experimental colitis[4], indicating a possible role of this chemokine axis in intestinal inflammatory response.

In UC patients, increased number of apoptotic epithelial cells is detected throughout the crypt[18]. Local massive apoptosis of epithelial cells disturbs the epithelial barrier, which can facilitate the infiltration of inflammatory cells, and aggravate mucosal damage[19]. Whether the CXCL12/CXCR4 chemokine axis plays a role in epithelial apoptosis and barrier function, however, needs to be further defined.

In the present study, we firstly assessed the effect of a CXCR4 antagonist, AMD3100, on dextran sulfate sodium (DSS)-induced colitis in mice. Morphology, colonic cytokines, myeloperoxidase (MPO) activity (an indicator of inflammatory infiltration), gut permeability, and epithelial apoptosis were all examined in the first series. To further elucidate the role of the CXCL12/CXCR4 interaction in colonic inflammation, we also investigated the effect ofAMD3100 on isolated peripheral blood mononuclear cells (PBMCs). In this series, cell migration and cytokine production were assessed.

MATERIALS AND METHODS

Animals

Female BALB/c mice (9 wk of age, weighing 20-22 g) were obtained from the Animal Facility of the Jinling Hospital (Nanjing, China). Animals were housed under controlled temperature, humidity and day-night cycles, with free access to standard laboratory feed and water. The Animal Studies Ethics Committee of Jinling Hospital approved all of the experiments.

Experimental design

For the induction of colitis, mice were given 5% DSS (5000 daltons; obtained from Wako Pure Chemical Industry, Japan) in their drinking water for 7 d. Normal control mice received regular drinking water throughout the experiment. The CXCR4 antagonist, AMD3100, was obtained from Sigma (St. Louis, MO, USA). Twenty-five micrograms of AMD3100 dissolved in 200 μL of phosphate-buffered saline (PBS) or 200 μL of PBS alone were administered intraperitoneally once daily during the study period. Eight mice were studied in each experimental group. On day 8, all mice were anesthetized with intraperitoneal administration of ketamine (50 mg/kg) and acepromazine (2 mg/kg), and the intestinal segments from the ileocecal valve to the anus (5-6 cm in length) were collected for subsequent assays.

Morphological examination

The distal segments of the colon (2 cm from the anal verge) were washed in PBS, fixed in 10% neutral buffered formalin, and embedded in paraffin wax. Five micrometers sections were deparaffinized with xylene, stained with hematoxylin and eosin, and examined by 2 experienced pathologists in blinded fashion. The following morphological criteria were considered: score 0, no damage; score 1 (mild), focal epithelial necrosis; score 2 (moderate), diffuse necrosis of the villi; score 3 (severe), necrosis with neutrophil infiltrate in the submucosa; score 4 (highly severe), widespread necrosis with massive neutrophil infiltrate and hemorrhage[1].

Measurement of colonic cytokines and MPO

The colonic levels of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interferon-γ (IFN-γ) were evaluated using commercial colorimetric kits (Jingmei Biotech, Beijing, China) according to the manufacturer's instructions. The tissue homogenate enzyme-linked immunosorbent assay was determined with respect to the concentration of protein, and expressed as pg/mg tissue protein.

Sequestration of leukocytes within the colon was evaluated by measuring tissue MPO activity[2]. Briefly, tissue samples were homogenized with 0.5% hexadecyltrimeth-

ylammonium bromide (Sigma, ST. Louis, MO, USA) in 50 mmol/L phosphate buffer (pH 6.0). The homogenate was sonicated for 10 s, freeze-thawed 3 times, centrifuged at 14000 rpm for 15 min, and the resulting suspension was used for assay. The assay mixture consisted of 20 μL of supernatant, 10 μL of tetramethylbenzidine (final concentration 1.6 mmol/L), and 70 μL of H_2O_2 (final concentration 3.0 mmol/L). MPO activity was assessed photometrically at 630 nm. The results were expressed with respect to the concentration of protein, and expressed as the activity per mg protein of the tissue (U/mg protein).

Gut permeability measurement

Intestinal permeability was assessed by the mucosal-to-serosal clearance of fluorescein isothiocyanate-conjugated dextran (4000 daltons, FD4; obtained from Sigma, St.Louis, MO, USA) in everted gut sacs, as described in previous studies[20,21]. Intestinal segments from the ileocecal valve to the anus were excised, and prepared in ice-cold modified Krebs-Henseleit bicarbonate buffer (KHBB contained in mmol/L: HEPES 10, NaCl 137, KCl 5.5, $NaHCO_3$ 4.2, Na_2HPO_4 0.3, $KHPO_4$ 0.4, $MgSO_4$ 0.4, $MgCl_2$ 0.5, $CaCl_2$ 1.3, glucose 19.5). The intestinal segment first underwent lavage with 3 mL of PBS to remove fecal material, and was then closed at one end with a 4-0 silk ligature. The gut sac was everted using a thin metal rod, then connected to a 1 mL syringe containing 0.4 mL of the KHBB solution, and secured with a 4-0 silk ligature 4 cm from the tip. The everted gut sac was gently distended with 0.4 mL of KHBB, suspended in a 100 mL beaker containing FD4 (20 μg/mL) in KHBB, maintained at 37℃ in a water bath, and continuously bubbled with a gas mixture containing 95% oxygen and 5% CO_2.

At the beginning of the incubation, a 1 mL sample was withdrawn from the beaker to determine the initial external (mucosal surface) FD4 concentration (FD4muc). After a 30-min incubation period, the gut sac was removed from the beaker, its diameter (D) and length (L) were measured, and the fluid on the serosal side was aspirated into the syringe to determine the FD4 concentration (FD4ser). The serosal and mucosal samples were centrifuged for 10 min at 1000 *g*. One hundred microliters of the supernatant was diluted with PBS (900 μL), and fluorescence was measured (λex = 492 nm, slit width = 1.5 nm; λem = 515 nm, slit width = 10 nm) in a spectrofluorometer (model F-7000, Hitachi, Japan). The mucosal-to-serosal clearance of FD4 was calculated using the following equations:

Mucosal surface area (A) = πLD.

Mass of FD4 in the gut sac after 30 min incubation (M) = (FD4ser) × 0.4.

Mucosal-to-serosal permeation rate of FD4 (PR, ng/min) = M/30 min.

Mucosal-to-serosal clearance of FD4 (C, nL/min per cm^2) = (PR/FD4muc)/A.

Assessment of epithelial apoptosis

Apoptosis of the colonic epithelium was assessed using a *nuclear* stain Hoechst-33342 (Sigma, St.Louis, MO, USA). Briefly, tissue sections (5 μm) were dewaxed with xylene and graded alcohols, and finally washed in tap water. Endogenous peroxidase activity was blocked by 3% (v/v) H_2O_2, and the antigen was recovered by microwave in 0.1 mol/L PBS (pH 7.2). Sections were immersed in Hoechst dye (working concentration 5 μg/mL) for 5 min, and then washed in PBS, 5 min for 3 times. The signals were visualized by a fluorescence microscope (model IX71, Olympus, Japan), and the number of positive cells/high power field was counted in 5 fields for each section as described by Cuzzocrea *et al*[22].

Isolation and stimulation of PBMCs

For isolation of PBMCs, 12 normal mice were anesthetized with intraperitoneal administration of ketamine (50 mg/kg) and acepromazine (2 mg/kg). Blood samples were obtained from the inferior cava vein by direct puncture. PBMCs were isolated by Ficoll-Hypaque plus density gradient centrifugation. The isolated PBMCs were resuspended in F-12K Nutrient Mixture (Invitrogen, Carlsbad, USA) with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 100 ng/mL phytohemagglutinin (PHA, activator of PBMCs; obtained from Sigma, St.Louis, MO, USA). Six hours after resuspension, the PBMCs were split into 3 groups (1.5×10^6 cells/mL). Group 1 was cultured in medium with PBS. Group 2 was re-stimulated with 50 ng/mL CXCL12 (Chemicon, MA, USA), and group 3 was pre-treated with 20 μmol/L AMD3100 (30 min before re-stimulation), and then treated with 50 ng/mL CXCL12. The cells were incubated for 24 h, and the cytokine production in supernatant was measured using a colorimetric commercial kit (Jingmei Biotech, Beijing, China) according to the manufacturer's instructions.

For assessment of cell migration, a 24-well transwell migration chamber with 8 μm pores (Greiner Bio-One, Frickenhausen, Germany) was used[23]. The isolated PBMCs was resuspended in serum-free medium with 0.2% bovine serum albumin to an appropriate concentration (1×10^6 cells/mL), then placed in the top chambers of the transwell (200 μL/well). The lower chambers were filled with 600 μL of control medium (containing 2.5% fetal bovine serum) with or without 50 ng/mL of CXCL12. Some of the cells were preincubated with the CXCR4 antagonist AMD3100 (20 μmol/L) for 30 min before being placed in the top chambers. Twelve hours after incubation, PBMCs that had not migrated to the lower chambers were removed from the upper surface of the transwell membrane with a cotton swab. Migrating cells on the lower membrane surface were fixed with methanol, stained with 10% Giemsa, and photographed under a light microscope. Values of integrated optical density (IOD) from the pictures were employed to assess the migration of PBMCs.

Statistical analysis

Results are presented as mean and standard error of the mean ± SE. One-way repeated-measures ANOVA (followed by multiple pair-wise comparisons using the Student-Newman-Kleus method) were used for the analysis of differences between the experimental and control groups. All statistical analyses were carried out using the SPSS version 11.5 for Windows (Chicago, IL, USA), with statistical significance set at $P < 0.05$.

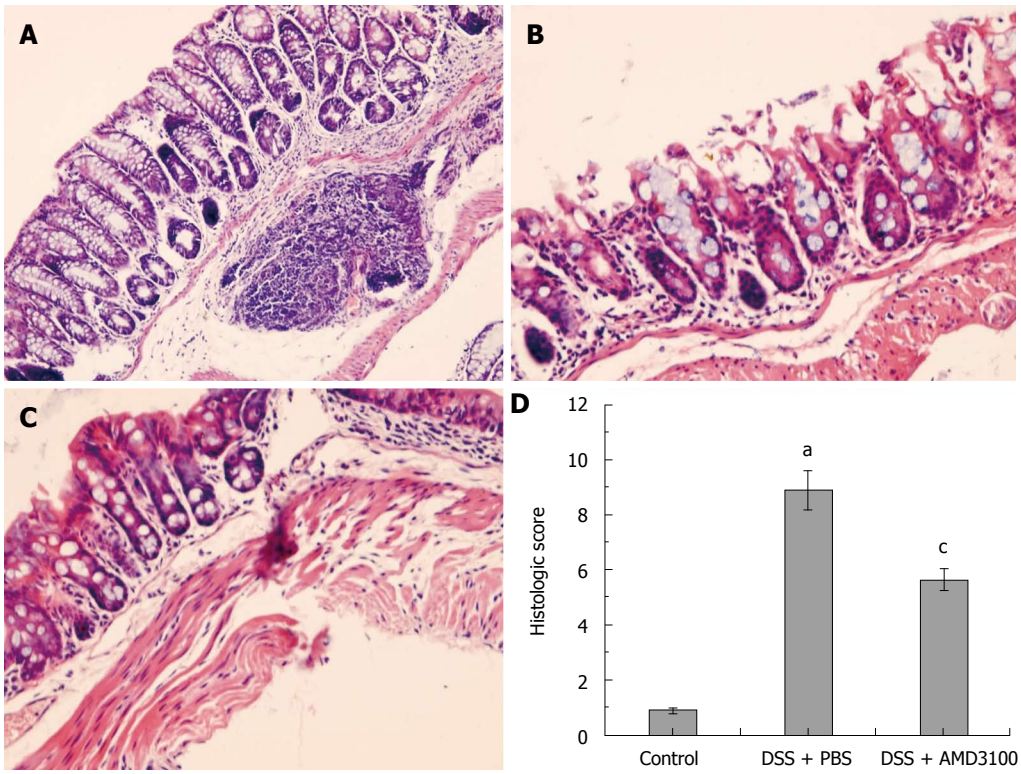


Figure 1 Effects of a chemokine stromal cell-derived factor-1 receptor (CXCR4) antagonist on mucosal damage in mice. Colitis was induced by administration of 5% dextran sulfate sodium (DSS) for 7 d. A: Common colonic morphologic characteristics are observed in tissue sections from control groups; B: Marked colonic injury is observed in tissue sections from the colitis group; C: Treatment with CXCR4 antagonist, AMD3100, significantly attenuates colonic damage; D: The histological score in mice. Eight mice were studied in each experimental group. Results are mean ± SE. $^{a}P < 0.05$ *vs* control group; $^{c}P < 0.05$ *vs* DSS + PBS group. Original magnification, × 200.

RESULTS

CXCR4 antagonist attenuated colonic mucosal damage in DSS-induced colitis

After induction of colitis with DSS, the colonic mucosa showed congestion, erosion, and hemorrhagic ulcerations. Histological findings demonstrated marked epithelial destruction, inflammatory infiltration, crypt distortion, and submucosal edema (Figure 1B). In AMD3100-treated mice, epithelial destruction, inflammatory infiltration, and submucosal edema were markedly reduced as compared with nontreated mice (Figure 1C). No histological alteration was observed in the intestinal segments from control mice (Figure 1A). As a result, the histological score in mice with DSS-induced colitis was significantly higher than that in control mice, and treatment with AMD3100 markedly reduced the histological score in mice with colitis (Figure 1D).

CXCR4 antagonist reduced colonic MPO and proinflammatory cytokines in DSS-induced colitis

Induction of DSS-induced colitis was assessed by histology and local cytokine production. In the present study, we also evaluated the effect of AMD3100 on colonic inflammation. As shown in Figure 2A-C, colonic TNF-α, IL-6, and IFN-γ levels were significantly elevated at 7 d after induction of colitis, as compared with control mice; treatment with AMD3100 markedly reduced colonic cytokines production in mice with colitis.

MPO activity, as a marker of local leukocyte sequestration, was also examined in this study. Figure 2D shows that colonic MPO activity was significantly increased 7 d after induction of colitis; treatment with AMD3100 decreased the colonic MPO level. These findings indicated that AMD3100 could inhibit the recruitment of inflammatory cells into the colonic segment.

CXCR4 antagonist enhanced colonic epithelial barrier in DSS-induced colitis

The mucosal-to-serosal clearance of permeability probe FD4 in everted gut sacs was measured to assess the epithelial barrier. The mucosal-to-serosal passage of FD4 was low in control mice, and the calculated clearance was 9.10 ± 1.10 nL/min per cm^2. DSS-administered mice demonstrated a marked increase in gut permeability, with the calculated clearance reaching 24.18 ± 1.83 nL/min per cm^2. In AMD3100 treated mice, there was a significant reduction in permeability, and the calculated clearance was 14.71 ± 1.43 nL/min per cm^2 (Figure 2E). These findings demonstrated that AMD3100 could enhance the colonic epithelial barrier.

CXCR4 antagonist ameliorated colonic epithelial apoptosis in DSS-induced colitis

According to the manufacturer of Hoechst-33342, the *nuclei* of apoptotic cells stain bright white, and are accompanied by apoptotic fragments, while the *nuclei* of live

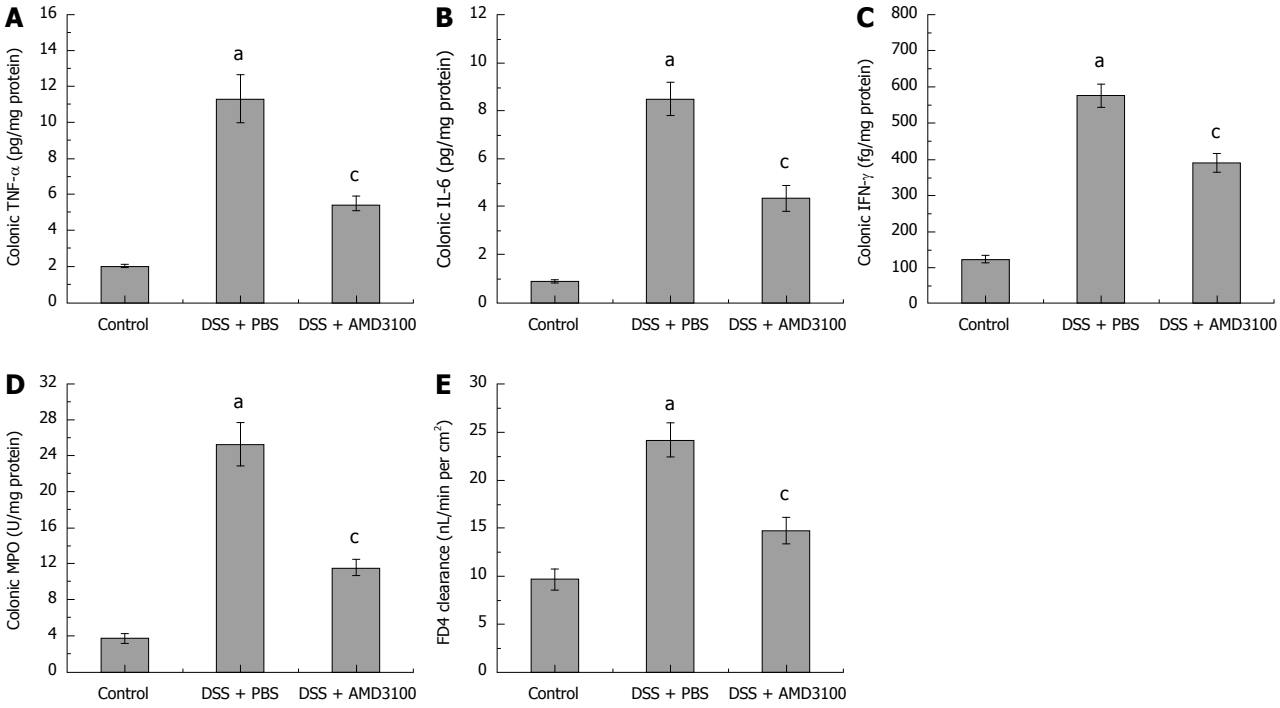


Figure 2 Effects of CXCR4 antagonist on colonic myeloperoxidase (MPO), proinflammatory cytokines, and gut permeability in mice. Colitis was induced by administration of 5% DSS for 7 d. Colonic levels of tumor necrosis factor-α (TNF-α) (A), interleukin-6 (IL-6) (B), interferon-γ (IFN-γ) (C), MPO (D), and FD4 clearance (E) are shown. Eight mice were studied in each experimental group. Results are mean ± SE. $^{a}P < 0.05$ *vs* control group; $^{c}P < 0.05$ *vs* DSS + PBS group.

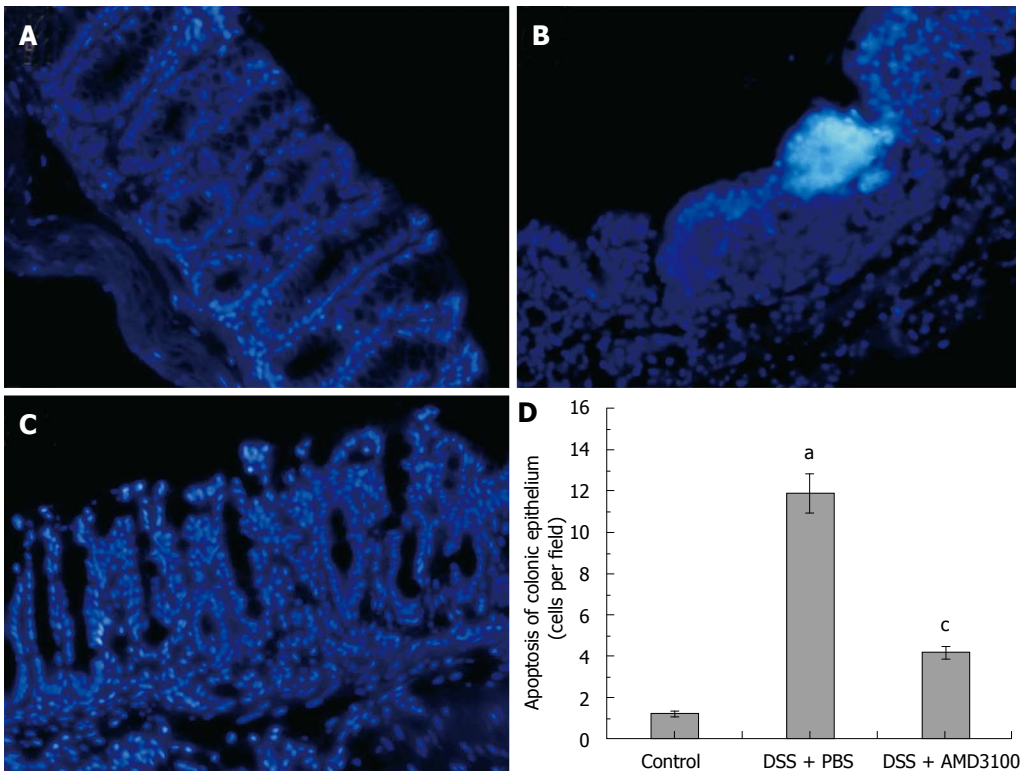


Figure 3 Effects of CXCR4 antagonist on colonic epithelial apoptosis in mice. Colitis was induced by administration of 5% DSS for 7 d. Tissue sections were stained with Hoechst-33342. A: Sporadic apoptotic cells were observed in intestinal segments from the control group; B: Clustered apoptotic cells were observed in tissue sections from the colitis group; C: Treatment with CXCR4 antagonist AMD3100 obviously decreased epithelial apoptosis; D: The numbers of apoptotic cells in intestinal segments. Eight mice were studied in each experimental group. Results are mean ± SE. $^{a}P < 0.05$ *vs* control group; $^{c}P < 0.05$ *vs* DSS + PBS group. Original magnification, × 200.

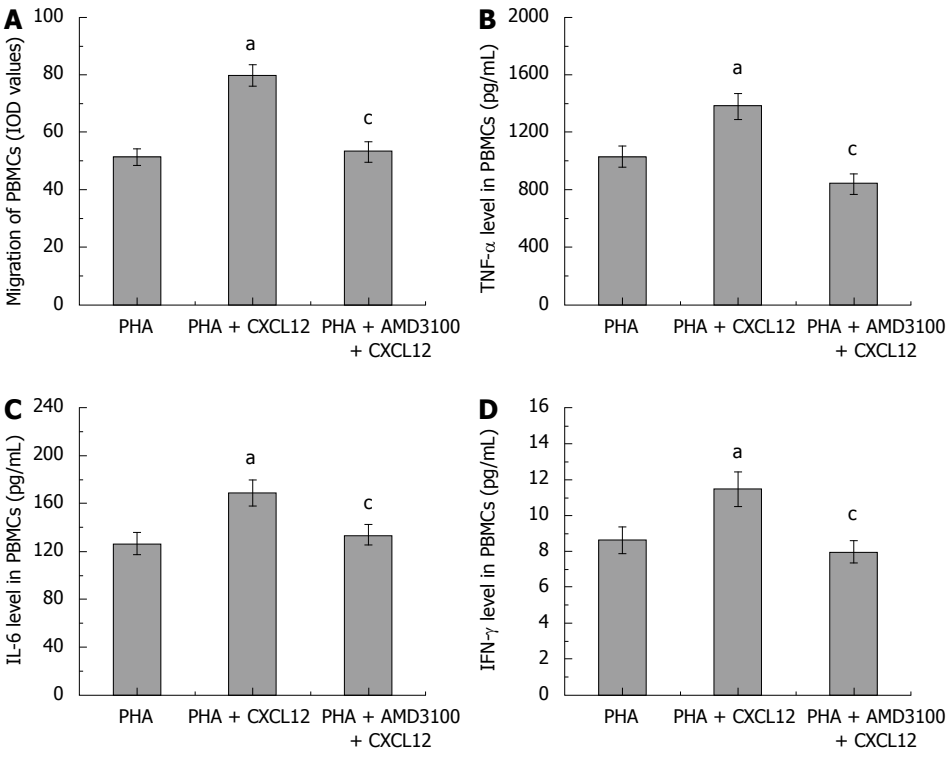


Figure 4 Effects of CXCR4 antagonist on migration and cytokine production of isolated PBMCs. To mimic the *in vivo* inflammatory response, the PBMCs were pre-activated with PHA. Migration of PBMCs (A) and levels of TNF-α (B), IL-6 (C), and IFN-γ (D) in PBMCs are shown. Six wells were studied in each experimental group. Results are mean ± SE. $^{a}P < 0.05$, chemokine stromal cell-derived factor-1 (CXCL12) group *vs* control group; $^{c}P < 0.05$, AMD3100 group *vs* CXCL12 group.

cells are stained as a uniform blue color. As in Figure 3, sporadic apoptotic cells were observed in the intestinal segments from control mice (apoptotic cells were 1.25 ± 0.12 per field). The number of apoptotic cells in the intestinal segments was markedly increased 7 d after induction of colitis, the apoptotic bodies mainly localized in the epithelium (apoptotic cells were 11.98 ± 0.81 per field). However, the presence of apoptotic cells was significantly decreased in the intestinal segments from AMD3100 treated mice (apoptotic cells were 4.23 ± 0.28 per field).

CXCR4 antagonist inhibited migration and cytokine production of isolated PBMCs

To evaluate whether AMD3100, the CXCR4 antagonist, could block PBMC migration, we performed an *in vitro* PBMCs chemotaxis assay. To mimic the *in vivo* inflammatory response, the PBMCs were pre-activated with PHA. The IOD values were employed to assess the number of migrated PBMCs. Figure 4A shows that CXCL12 treatment significantly increased the migration of PBMCs, as compared with the control group, the IOD values being 79.68 ± 3.76 and 51.28 ± 2.97, respectively, and the migration of PBMCs were inhibited by pretreatment with AMD3100 (IOD values 53.27 ± 3.70).

We also measured the cytokine production from isolated and PHA pre-activated PBMCs. As shown in Figure 4B-D, the supernatant levels of TNF-α, IL-6, and IFN-γ from CXCL12-treated PBMCs were moderately higher than that in the control group, and AMD3100 pre-treatment could attenuate the increase in TNF-α, IL-6, and IFN-γ in PBMCs.

DISCUSSION

The present study demonstrated that administration of the CXCR4 antagonist, AMD3100, decreased the severity of DSS-induced colitis in mice, as assessed by morphology, colonic cytokines, epithelial apoptosis, and gut permeability. More importantly, the CXCR4 antagonist also inhibited the migration and cytokine production of activated PBMCs. These results strongly suggest that the CXCR4 antagonist has a therapeutic effect on experimental colitis.

The CXCL12/CXCR4 axis is an efficacious leukocyte chemoattractant, which can attract lymphocytes and mononuclear cells from the bloodstream to the site of inflammation[24,25]. A previous study demonstrated that CXCL12 could promote natural killer cell migration into tissues by stimulating the production of matrix metalloproteinase-1 and subsequent degradation of the extracellular matrix proteins[26]. By provoking activation of protein kinase C, CXCL12 also induced adhesion of monocytes to human umbilical vein endothelial cells[25]. In patients with bursitis or acute lung injury, increased local CXCL12 and CXCR4 expression was associated with aggravated tissue damage, which could be attenuated by CXCL12 or CXCR4 blockade, suggesting a role of the CXCL12/CXCR4 chemokine axis in local inflammation[12-14,27].

Recently, several studies have been performed to investigate the role of the CXCL12/CXCR4 chemokine axis in IBD, particularly in UC. Katsuta *et al*[28] have previously demonstrated that the expression of CXCL12 mRNA in colonic mucosa of UC patients was higher than that in patients with Crohn's disease. Dotan *et al*[17] also reported that CXCL12 and CXCR4 were constitutively expressed on human intestinal epithelial cells (IECs), and were up-regulated in IBD IECs; $CXCR4^{+}$ cells were increased in the lamina propria of IBD patients, and antibodies against CXCR4 or CXCL12 significantly blocked the migration of

lamina propria and peripheral blood T cells, identifying an inflammatory role for the CXCL12/CXCR4 axis in the intestinal mucosa. In patients with refractory UC, increased CXCR4 expression was also observed on peripheral blood T cells[16]. In DSS-induced colitis, both CXCR4 expression on leukocytes and CXCL12 expression in colonic tissue were significantly increased; administration of a CXCR4 antagonist TF14016 ameliorates colonic inflammation in DSS-induced colitis and IL-10 knockout mice, and also reduced TNF-α and IFN-γ production in mesenteric lymph node cells, indicating a possible role of this chemokine axis in the pathophysiology of UC[4].

Consistent with previous reports, our present study also demonstrated marked mucosal damage and inflammatory responses in DSS-induced colitis. MPO activity, as an indicator of inflammatory infiltration, was significantly elevated 7 d after induction of colitis, in parallel with increased colonic TNF-α, IL-6, and IFN-γ. Administration of the CXCR4 antagonist, AMD3100, decreased the histological score, colonic cytokines, and inflammatory infiltration in DSS-induced colitis in mice. These results are in agreement with a previous report that the CXCR4 antagonist, TF14016, could also ameliorate DSS-induced colitis[4].

The intestinal epithelium is a single cell layer that forms the barrier between the body and the luminal gastrointestinal contents[29]. The central role of the intestinal epithelium is to limit access of toxins and microbes to underlying tissues, and intestinal barrier dysfunction has strong potential to result in IBD[30]. Accordingly, our results demonstrated that mice subjected to DSS developed significant intestinal barrier failure, as evidenced by a marked increase in gut permeability to FD4, while AMD3100 administration preserved the intestinal barrier function by reducing gut permeability. Although there is an unanswered question regarding IBD as to whether dysfunction of the intestinal barrier is a primary contributor to inflammation, or is a consequence of the action of inflammatory mediators[31], our present study clearly showed that AMD3100 treatment reduced both the inflammatory response and gut permeability in mice with colitis, identifying a therapeutic effect of the CXCR4 antagonist in experimental colitis.

A previous study also demonstrated that the maintenance of the intestinal epithelium was mainly dependent upon the regulation of proliferation and apoptosis[32]. Local massive apoptosis of epithelial cells would disturb the epithelial barrier, facilitate the infiltration of inflammatory cells, and aggravate mucosal damage[19,33]. In the present study, we found that the number of apoptotic cells in the colonic epithelium was markedly increased in DSS-induced colitis in mice. This was consistent with a previous study which demonstrated that apoptotic epithelial cells in the crypt of UC patients were much more abundant than in the control group[18]. Our present study also reported that AMD3100 treatment significantly decreased the presence of apoptotic cells in intestinal segments of DSS-induced colitis in mice, indicating that the CXCL12/CXCR4 axis also plays a role in epithelial apoptosis during the UC.

The infiltration of leucocytes into the colonic mucosa is believed to play an important role in tissue damage and clinical symptoms in both human and experimental UC[2,3]. Thus, in the present study, we further clarified the effect of the CXCR4 antagonist on migration and cytokine production of PBMCs. To mimic the *in vivo* inflammatory response, the PBMCs were pre-activated with PHA. We demonstrated that, although CXCL12 increased PBMC migration, the CXCR4 antagonist significantly attenuated the migration of PBMCs. In addition, AMD3100 moderately decreased cytokine production in PBMC culture. Taken together, the present data suggest that the amelioration of DSS-induced colitis by the CXCR4 antagonist can be mainly attributed to its effect on migration of PBMCs.

In conclusion, the present study demonstrated that the CXCR4 antagonist, AMD3100, decreased the severity of DSS-induced colitis in mice, by inhibiting the migration and cytokine production of activated PBMCs, and identified a therapeutic effect of the CXCR4 antagonist in experimental colitis. These data also suggested a pivotal role of the CXCL12/CXCR4 chemokine axis in the pathogenesis of UC.

COMMENTS

Background

Ulcerative colitis (UC) is characterized by frequent diarrheal attacks and anal bleeding. Histologic characteristics of UC are the invasion of the crypt epithelium and lamina propria by peripheral blood mononuclear cells (PBMCs), disruption of the epithelial lining, and consequently mucosal ulceration and crypt abscess formation in the bowel wall. Regulation of the migration of inflammatory leukocytes into the intestinal tissues is considered to be a therapeutic option for patients with UC.

Research frontiers

Chemokine stromal cell-derived factor-1 receptor (CXCR4) is specific receptor for chemokine chemokine stromal cell-derived factor-1 (CXCL12), and the latter is a potent chemoattractant for PBMCs. The expression of CXCL12 and CXCR4 on intestinal epithelial cells, lamina propria T cells and PBMCs are significantly increased in UC patients, and block of CXCR4 ameliorates the colonic inflammation in experimental colitis. Whether a CXCR4 antagonist enhances epithelial barrier function, however, has not been unequivocally addressed.

Innovations and breakthroughs

A recent report has highlighted the importance of CXCR4 in colonic inflammation of experimental colitis. Whether CXCR4 plays a role in the epithelial barrier is so far uncharacterized. This is the first study to report that, in addition to inflammation inhibition, the CXCR4 antagonist, AMD3100, also decreased epithelial apoptosis and gut permeability in experimental colitis, and consequently enhanced the epithelial barrier function.

Applications

By understanding the role of CXCR4 in colonic inflammation and epithelial barrier, this study may represent a future strategy for therapeutic intervention in the treatment of patients with UC.

Peer review

The authors present a concise argument for conducting the study which is the participation of the lymphocyte CXCR4 receptor in the augmentation of the immune response found in ulcerative colitis. Their contention that blocking this receptor with an experimental CXCR4 antagonist would ameliorate dextran sulfate sodium induced colitis was demonstrated by using the specific CXCR4 blocking agent known as AMD3100.The experimental model is well thought out and the conclusions are justified by the results obtained. The discussion is adequately developed and focused on the experimental results.

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ORIGINAL ARTICLE

Expression and significance of TLR4 and HIF-1α in pancreatic ductal adenocarcinoma

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Abstract

AIM: To investigate the expression of toll-like receptor (TLR) 4, nuclear factor-κB (NF-κB) p65 and hypoxia-inducible transcription factor 1α (HIF-1α) in pancreatic ductal adenocarcinoma and their clinical significance.

METHODS: The mRNA of TLR4 and HIF-1α were investigated by real-time polymerase chain reaction in 30 cases of pancreatic ductal adenocarcinoma and its adjacent tissues, and expression of TLR4, NF-κB p65 and HIF-1α protein were detected by immunohistochemistry in 65 cases of pancreatic ductal adenocarcinoma tissues and 38 cases of corresponding adjacent tissues. The relationship between TLR4 or HIF-1α and pathologic features, as well as the association between TLR4 and HIF-1α, were also analyzed. Kaplan-Meier method was used to assess the impact of expression of TLR4 and HIF-1α on survival of patients with pancreatic cancer.

RESULTS: The relative quantification of TLR4 and HIF-1α mRNA in tumor tissues was 0.81 ± 0.10 and 0.87 ± 0.11, respectively, significantly higher than that in adjacent tissues (0.81 ± 0.10 *vs* 0.70 ± 0.16, $P = 0.002$; 0.87 ± 0.11 *vs* 0.68 ± 0.13, $P = 0.000$). The protein expression of TLR4, NF-κB p65 and HIF-1α in tumor tissues was 69.20%, 66.15% and 70.80%, respectively, being significantly higher than that in adjacent normal tissues (69.20% *vs* 39.50%, $P = 0.003$; 66.15% *vs* 31.58%, $P = 0.001$; 70.80% *vs* 36.80%, $P = 0.001$). There was no significant correlation between TLR4 or HIF-1α expression and the age, gender, tumor location, the degree of tumor differentiation in the patients ($P > 0.05$). However, there was significant correlation between the expression of TLR4 or HIF-1α and tumor size, lymph node metastasis, venous invasion and clinical staging ($P < 0.05$). The expression of TLR4 and HIF-1α had a significant impact on survival of patients with pancreatic adenocarcinoma.

CONCLUSION: TLR4, NF-κB p65 and HIF-1α are overexpressed in pancreatic adenocarcinoma, TLR4 may be partly involved in up-regulating HIF-1α, and both synergestically promote development of pancreatic adenocarcinoma.

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Key words: Pancreatic ductal adenocarcinoma; Toll-like receptor 4; Nuclear factor-κB p65; Hypoxia-inducible factor 1

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INTRODUCTION

Because of pancreas insidious nature and anatomic location, pancreatic ductal adenocarcinoma (PDAC) is difficult to be detected early, often becomes advanced by the time of diagnosis. The 5-year survival rate is less than 5% and a median survival time is 5-6 mo[1]. Although 10%-15% of patients underwent potentially surgical resection, the median survival was only 10-18 mo with a 5-year survival of 17%-24%[2]. Most failures occur within 1-2 years after surgery because of local recurrence, hepatic metastases, or both[3]. Therefore, understanding the molecular biology of pancreatic cancer is important for diagnosis, prevention and treatment of PDAC.

Toll-like receptors (TLRs) are a family of pattern recognition receptors that are best-known for their role in host defense against infection. In human, 13 types of TLRs have been identified, mainly expressed by immune cells and epithelial cells. Recently, TLRs have also been detected in many tumor cell lines or tumors, especially epithelium-derived cancers[4]. TLRs may promote the proliferation and inhibit the apoptosis, and lead to migration, invasion and angiogenesis of tumor[5]. However, it remains unknown whether TLR4 is expressed and what role it plays in PDAC.

Hypoxia-inducible transcription factor 1 (HIF-1) is a heterodimer that consists of hypoxic response factor HIF-1α and the constitutively expressed HIF-1β[6]. HIF-1α is unstable in well-oxygenated tissues due to ubiquitin-mediated degradation, but rapidly becomes stable in hypoxic conditions[7]. In human cancers, HIF-1α is overexpressed as a result of intratumoral hypoxia and genetic alterations[8], HIF-1 targets the transcription of over 60 genes involved in many aspects of cancer biology including cell survival, glucose metabolism, cell invasion and angiogenesis[9]. However, the regulatory mechanisms of HIF-1α in tumor microenviroment are complex and still not fully understood. Recently, some studies have demonstrated that bacteria or Lipopolysaccharide (LPS)-induced HIF-1α stabilization even under normoxic conditions[10-12], and LPS-induced HIF-1α accumulation was TLR4 dependent[13] in immune cells, such as macrophage[10,12,13], monocytes[11]. Thus, we wonder whether there is some association between HIF-1α and TLR4 in tumor cells similar to immune cells.

In this study, TLR4 and HIF-1α mRNA and protein were detected in PDAC and its adjacent tissues by real-time polymerase chain reaction (PCR) and immunohistochemical methods. The association between TLR4 and HIF-1α, and impact of their expression on patients' survival were analyzed. The nuclear factor-κB (NF-κB) p65, which is an essential component in both TLR4 signal pathway and HIF-1 signal pathway, was also detected by immunohistochemistry.

MATERIALS AND METHODS

Patients and samples

The study group consisted of 65 patients with primary PDAC, 40 males and 25 females, ranged in age from 36 to 82 years, with a median age of 52 years. They underwent pancreaticoduodenectomy or distal pancreatectomy with lymph node dissection at Pancreatic Center of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, between January 2007 and December 2008. Sixty-five cases of pancreatic cancerous tissues and 38 cases of corresponding adjacent normal tissues were collected, the adjacent normal tissues were taken from the edge of the tumor and confirmed by pathology. All samples were divided into two parts immediately after removed from patients, one was put into liquid nitrogen for real-time PCR, the other was fixed in 4% buffered formaldehyde and embedded in paraffin wax for immunohistochemistry. None of the patients received preoperative chemotherapy or radiation therapy. Among 65 patients, 25 (38.5%) cases were moderately differentiated, 21 (32.3%) well differentiated, and 19 (29.2%) poorly differentiated. Forty cases (61.5%) had lymph node metastases. Nineteen cases (29.2%) had venous invasion. According to the sixth edition of the TNM classification of the International Union Against Cancer (UICC), 24 patients were at pathological stage Ⅰ or Ⅱ, and 41 patients were at stage Ⅲ or Ⅳ. All patients received chemotherapy routinely after operation, and were followed up regularly for 5-35 mo (mean, 14 mo). The procedure had been approved by the Ethics Committee of Union Hospital, Tongji Medical College.

RNA isolation, reverse transcription and real-time PCR

The total RNA of PDAC and adjacent tissues were extracted using Trizol (invitrogen, USA) according to the manufacturer's protocol. The concentration was analyzed by ultraviolet spectrophotometer. Two micrograms of RNA was used for the reverse transcription. TLR4, HIF-1α and β-actin genes were amplified with SYBR Green in a fluorescence reader FTC-2000 (Feng Ling Bio-engineering Co., Ltd., Shanghai, China). The primer sequences for TLR4 gene were as follows: 5'-ACCTGTCCCTGAACCCTATGAA-3' (forward) and 5'-CTTCTAAACCAGCCAGACCTTG-3' (reverse); the primer sequences for HIF-1α gene were: 5'-TGAAGTGTACCCTAACTAGCCG-3' (forward) and 5'-AATCAGCACCAAGCAGGTCATAG-3' (reverse); and the primer sequences for β-actin gene were: 5'-GAAACTACCTTCAACTCCATC-3' (forward) and 5'-CGAGGCCAGGATGGAGCCGCC-3' (reverse). Cycling conditions were as follows: initial denaturation at 94℃ for 5 min, followed by 30 cycles at 94℃ for 30 s, at 58℃ for 30 s and at 72℃ for 30 s. The negative control was performed using normal pancreatic tissue taken far away from the cancerous tissues. The results were analyzed by the fluorescence reader program [calculation formula: folds = $2^{-\Delta\Delta Ct}$, $\Delta\Delta Ct$ = [(Ct target gene - Ct β-actin) experiment sample - (Ct target gene - Ct β-actin) control sample][14]. Each experiment was carried out in triplicate

using β-actin as an internal standard; the results were expressed as mean ± SD of representative triplicates.

Immunohistochemistry

The tissue specimens were fixed in 10% formalin buffered at pH 7.0 for 24 h and paraffin-embedded. Histologic slides (5 μm) were deparaffined in xylene and rehydrated through a series of graded ethanol. Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide in methanol for 10 min. Antigen retrieval was performed by microwaving the slides in citrate buffer (pH 6.0) for 20 min at 120℃. Then the sections were washed and blocked with normal goat serum (Streptavidin/Peroxidase immunohistochemical kit, Zhongshan Goldenbridge Biological Technology Co., LTD, Beijing, China) for 30 min at room temperature, subsequently exposed to the primary antibody (rabbit polyclonal anti-TLR4, 1:50, Santa Cruz, CA, USA; mouse monoclonal anti-HIF-1α, 1:50, Santa Cruz, CA, USA; NF-κB p65 rabbit monoclonal antibody, 1:50, Cell Signaling Technology Inc, MA, USA). overnight at 4℃. After washing in phosphate buffered saline (PBS), the slides were incubated with a biotinylated goat anti-mouse or anti-rabbit secondary antibody (1:200, Streptavidin/Peroxidase immunohistochemical kit) for 30 min at room temperature. After reactions with freshly prepared diaminobenzidine-tetrahydrochloride (DAB kit, Zhongshan Goldenbridge Biological Technology Co., LTD, Beijing, China), the slides were counterstained in haematoxylin and coverslipped in a systemic mounting medium. Negative controls were prepared without the primary antibodies.

The assessment of TLR4, HIF-1α and NF-κB p65 was based on previously described guidelines[15], which consist of the percentage of stained tumor cells and the staining intensity. At least 3 different high-power (× 400) fields of tumor infiltration were examined. The percentage of positive tumor cells was rated as follows: 1 point, ≤ 10% positive tumor cells; 2 points, 11%-50% positive tumor cells; 3 points, 51%-80% positive tumor cells; and 4 points, ≥ 81% positive tumor cells. The staining intensity was rated as follows: 1 point, weak intensity; 2 points, moderate intensity; and 3 points, strong intensity. Points for expression and percentage of positive cells were added, and specimens were attributed to four groups according to their overall score: negative, ≤ 10% of cells stained positive, regardless of intensity; weak expression, 3 points; moderate expression, 4-5 points; and strong expression, 6-7 points. Weak expression was rated as negative, and moderate and strong expressions were rated as positive for analysis. Two independent investigators (Zhang JJ and Tian Y) blinded to clinical data performed the analysis. Specimens scored differently by the two investigators were reinvestigated together using a multiheaded microscope.

Statistical analysis

For statistical analysis, SPSS 15.0 was used. The independent *t* test was used to compare the means of TLR4 or HIF-1α mRNA between PDAC and its adjacent tissues. The χ^2 test was used to analyze the difference in expression of TLR4, NF-κB p65 and HIF-1α protein between PDAC and its adjacent tissues, as well as correlation between TLR4, HIF-1α and clinicopathologic features. One-way analysis of variance was used for three groups. Survival curves were calculated using the Kaplan-Meier method and analyzed using the log-rank test. Differences at $P < 0.05$ were considered to be statistically significant.

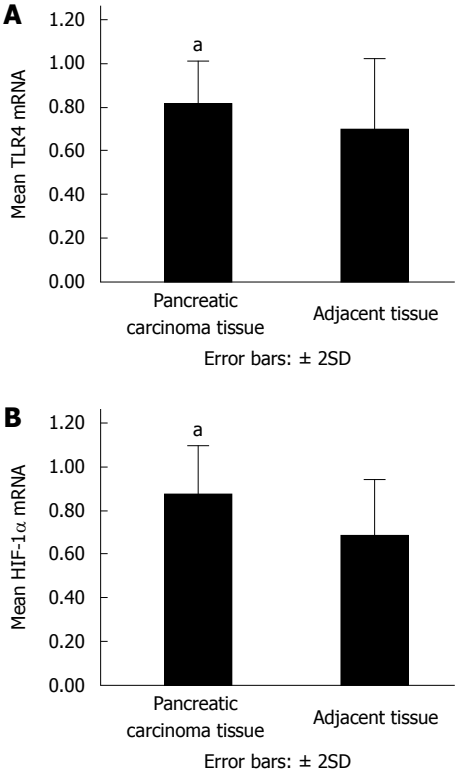


Figure 1 Toll-like receptor (TLR) 4 and hypoxia-inducible transcription factor-1α (HIF-1α) mRNA in pancreatic carcinoma and adjacent tissues. [a]*P* < 0.05 *vs* adjacent tissues. A: TLR4 mRNA in 30 cases of pancreatic carcinoma and adjacent tissues was determined by real-time polymerase chain reaction (PCR); B: HIF-1α mRNA in 30 cases of pancreatic carcinoma and adjacent tissues was determined by real-time PCR.

RESULTS

TLR4 is overexpressed in PDAC

To investigate whether TLR4 is expressed in PDAC, we first detected TLR4 mRNA by real-time PCR. The results showed that the mean level of TLR4 mRNA was 0.81 ± 0.10 in tumor tissues, and it was 0.70 ± 0.16 in adjacent normal tissues, with significant difference between them ($P = 0.002$, Figure 1A). We then detected TLR4 protein by immunohistochemistry, and found that TLR4 protein was localized in cytoplasm and membrane of tumor cells (Figure 2A and B), and the expression of TLR4 was 69.2% (45/65) in cancerous tissues, which was significantly higher than that in adjacent normal tissues (69.2% *vs* 39.5%, $P = 0.003$, Table 1). When analyzing the relationship between TLR4 and clinicopathological features, we found that the expression of TLR4 mRNA or protein was not correlated with age, gender, location and differentiation of the tumor ($P > 0.05$), but

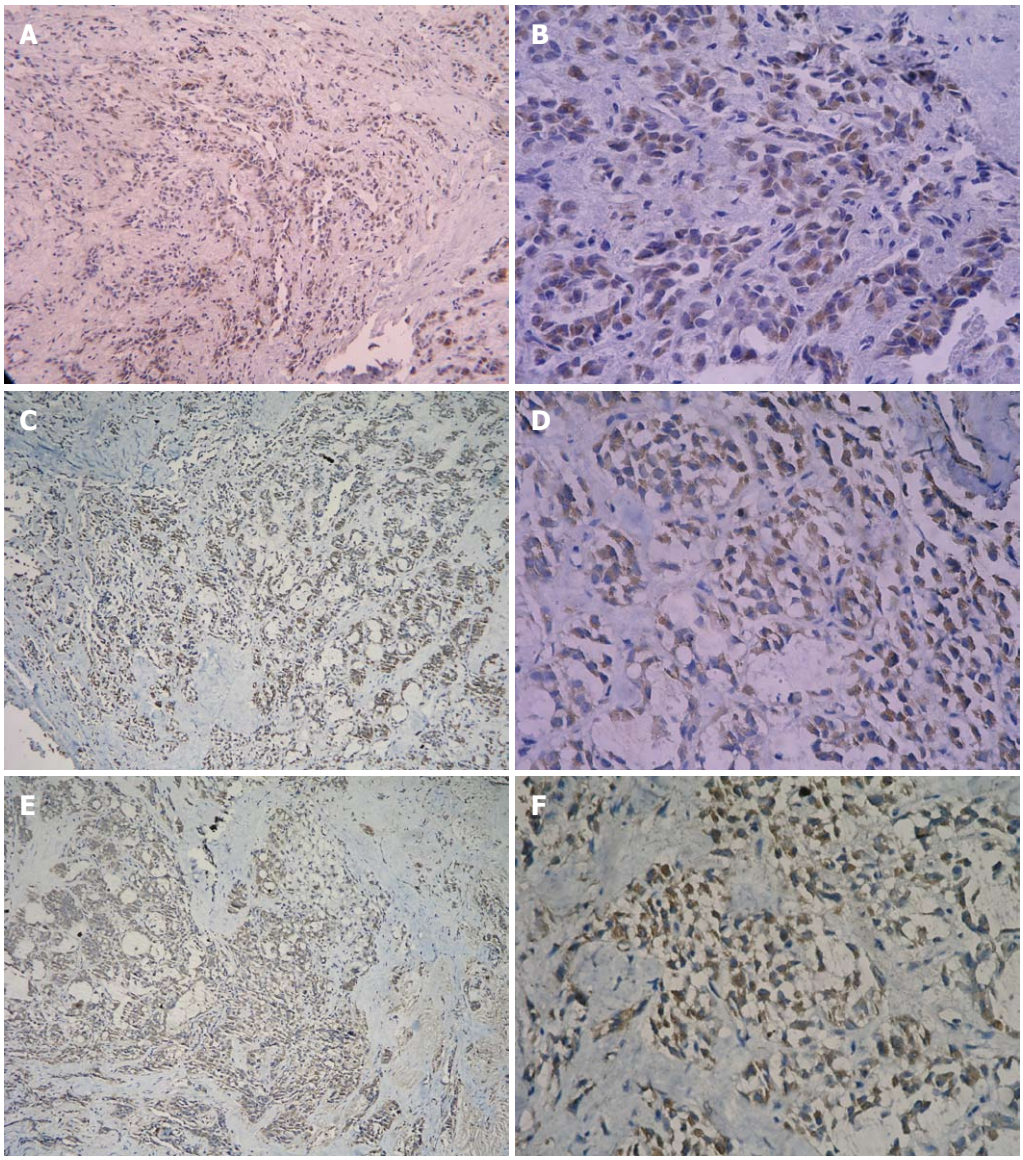


Figure 2 Expression of TLR4 and HIF-1α protein in pancreatic carcinoma tissue. Brown color displays the positive expression. A: Expression of TLR4 protein in carcinoma tissue (SP, × 100); B: Expression of TLR4 protein in carcinoma tissue (SP, × 400); C: Expression of HIF-1α protein in carcinoma tissue (SP, × 100); D: Expression of HIF-1α protein in carcinoma tissue (SP, × 400); E: Expression of NF-κB p65 protein in carcinoma tissue (SP, × 100); F: Expression of NF-κB p65 protein in carcinoma tissue (SP, × 400).

Table 1 Expression of TLR4 and HIF-1α protein in pancreatic carcinoma and adjacent tissues *n* (%)

	Cases	TLR4 protein		HIF-1α protein	
		Positive	*P* value	Positive	*P* value
Tumor tissues	65	45 (69.20)	0.003	46 (70.80)	0.001
Adjacent tissues	38	15 (39.50)		14 (36.80)	

TLR4: Toll-like receptor 4; HIF-1α: Hypoxia-inducible transcription factor-1α.

significantly correlated with tumor size, lymph node involvement, venous invasion and pathological stage ($P < 0.05$, Table 2).

HIF-1α is overexpressed in PDAC

We continued to detect HIF-1α in PDAC, and found that the mean level of HIF-1α mRNA in tumor tissues was 0.87 ± 0.11, being significantly higher than that in adjacent tissues, which was 0.68 ± 0.13 ($P = 0.000$, Figure 1B). Immunohistochemical results showed that HIF-1α was expressed in the nuclei and/or the cytoplasm of tumor cells (Figure 2C and D). The expression of HIF-1α was found in 46 of 65 (70.8%) cases of tumor tissues, and 14 of 38 (36.8%) cases of adjacent tissues, with significant difference between them (Table 1, $P < 0.05$). Furthermore, we also found that the expression of HIF-1α was correlated with tumor size, lymph node involvement, venous invasion and pathological stage ($P < 0.05$, Table 2).

Correlation of TLR4 and HIF-1α expressions in PDAC

In macrophages, LPS can raise the level of HIF-1α in a TLR4-dependent fashion[13], but it remains unknown in tumor cells. To study whether TLR4 is associated with HIF-1α in PDAC, we performed the correlative analysis.

Table 2 Relationship between TLR4, HIF-1α and clinical pathologic features

	TLR4 mRNA		TLR4 protein			HIF-1α mRNA		HIF-1α protein		
	mean ± SD	*P*	+	-	*P*	mean ± SD	*P*	+	-	*P*
Age (yr)		0.258			0.381		0.150			0.229
≤ 59	0.83 ± 0.10		24	13		0.90 ± 0.13		24	13	
≥ 60	0.79 ± 0.09		21	7		0.84 ± 0.08		22	6	
Gender		0.133			0.137		0.744			0.464
Male	0.79 ± 0.09		25	15		0.88 ± 0.11		27	13	
Female	0.84 ± 0.10		20	5		0.86 ± 0.12		19	6	
Location of tumor		0.374			0.280		0.197			0.680
Pancreas head	0.80 ± 0.11		31	11		0.89 ± 0.13		29	13	
Pancreas body-tail	0.83 ± 0.09		14	9		0.84 ± 0.08		17	6	
Histology		0.654			0.585		0.279			0.224
Well	0.84 ± 0.11		13	8		0.88 ± 0.11		12	9	
Moderate	0.80 ± 0.11		19	6		0.89 ± 0.12		20	5	
Poor	0.80 ± 0.07		13	6		0.82 ± 0.08		14	5	
Tumor size (cm)		0.021			0.014		0.030			0.039
≤ 2	0.77 ± 0.07		9	10		0.82 ± 0.08		10	9	
> 2	0.85 ± 0.10		36	10		0.91 ± 0.12		36	10	
pN		0.012			0.017		0.049			0.038
Positive	0.85 ± 0.09		32	8		0.90 ± 0.12		32	8	
Negative	0.77 ± 0.08		13	12		0.82 ± 0.08		14	11	
Venous invasion		0.021			0.023		0.018			0.033
Positive	0.85 ± 0.09		17	2		0.91 ± 0.12		17	2	
Negative	0.77 ± 0.09		28	18		0.82 ± 0.07		29	17	
pStage		0.031			0.010		0.016			0.024
Ⅰ, Ⅱ	0.77 ± 0.09		12	12		0.81 ± 0.07		13	11	
Ⅲ, Ⅳ	0.84 ± 0.09		33	8		0.91 ± 0.12		33	8	

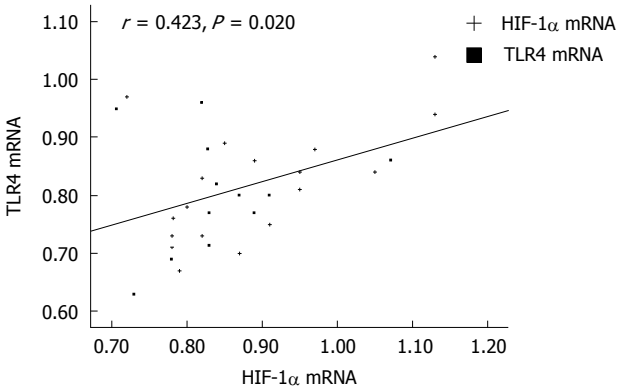


Figure 3 Correlation between TLR4 mRNA and HIF-1α mRNA.

The results showed that there existed a significant correlation between TLR4 mRNA and HIF-1α mRNA (r = 0.423, P = 0.020, Figure 3), similar result was also found between TLR4 and HIF-1α protein (r_p = 0.451, P < 0.05, Table 3), suggesting that TLR4 has a positive correlation with HIF-1α in PDAC.

NF-κB p65 is overexpressed in PDAC

NF-κB is an essential downstream component of TLR signal pathway[16], and HIF-1α is a target gene of NF-κB[17]. To further explore the association between TLR4 and HIF-1α, we investigated NF-κB p65 expression in PDAC by immunochemistry. The results showed that NF-κB p65 was expressed in the nuclei and/or the cytoplasm of tumor cells (Figure 2E and F), and the positive expression of NF-κB p65 was found in 43 (66.15%) of 65 cases of tumor tissues, being significantly higher than

Table 3 Correlation between TLR4 and HIF-1α protein

	TLR4 expression		r_p	*P* value
	Positive (n = 45)	Negative (n = 20)		
HIF-1α expression			0.451	0.000
Positive (n = 46)	38	8		
Negative (n = 19)	7	12		

in 12 (31.58%) of 38 cases of adjacent tissues (P = 0.001). Considering NF-κB is an essential component in TLR4 signal pathway and an important modulator of HIF-1α, we could speculate that NF-κB p65 may be a link in the association between TLR4 and HIF-1α.

Overexpression of TLR4 and HIF-1α in PDAC has a negative impact on patient survival

In order to understand whether over-expression of TLR4 and HIF-1α in PDAC has an impact on patient survival, the relationship between the expression of TLR4 or HIF-1α and life span of patients was analyzed. The mean survival of patients without over-expression of TLR4 in tumor tissues was 19.7 mo, and it was 12.4 mo in those with over-expression of TLR4, log-rank analysis showed there was a significant difference (P = 0.011, Table 4 and Figure 4A). Similarly, the mean survival of patients without over-expression of HIF-1α in tumor tissues was 20.1 mo, being significantly longer than those with over-expression of HIF-1α, which was 12.8 mo (P = 0.005, Table 4 and Figure 4B). In addition, the survival time of group with neither TLR4 nor HIF-1α over-expression

Table 4 Impact of TLR4 and HIF-1α on survival time

Protein	Expression	Estimated survival	95% CI		*P*
			Lower bound	Upper bound	
TLR4	Positive	12.4	10.8	14.0	0.011
	Negative	19.7	15.4	23.9	
HIF-1α	Positive	12.8	11.1	14.4	0.005
	Negative	20.2	15.2	25.1	
TLR4 positive	HIF-1α positive	11.8	10.1	13.4	0.132
	HIF-1α negative	15.0	10.9	19.2	
TLR4 negative	HIF-1α positive	16.5	12.5	20.6	0.089
	HIF-1α negative	21.6	15.1	28.2	0.014[1]

[1]*P* value compared between patients with both negative TLR4 and HIF-1α expression and those with both positive TLR4 and HIF-1α expression.

was 21.6 mo, significantly longer than those with both over-expressions, which was 11.8 mo ($P = 0.014$, Table 4 and Figure 4C).

DISCUSSION

TLR4 was studied first in TLRs, which is mainly expressed in immune cells and epithelial cells. The activation of TLR4 facilitates the activation of two pathways: the MyD88 (myeloid differentiation primary-response protein 88)-dependent and MyD88-independent pathways. The MyD88-dependent pathway involves the early phase of NF-κB activation, which leads to the production of inflammatory cytokines. The MyD88-independent pathway activates interferon (IFN)-regulatory factor (IRF3) and involves the late phase of NF-κB activation, both of which lead to the production of IFN-β and the expression of IFN-inducible genes. In 1998, TLR4 was identified as the signal transducer for LPS, a major cell-wall component of Gram-negative bacteria[18], in addition to microbial ligands, an increasing number of endogenous ligands are being reported as candidate stimulators of TLR4, including heat shock proteins (HSP60, HSP70, endoplasmin, HSPB8 and α-crystallin A chain), high mobility group box 1 (HMGB1), and various products of the extracellular matrix such as fibronectin, heparan sulphate, biglycan, fibrinogen, oligosaccharides of hyaluronan and hyaluronan breakdown fragments[5].

There are increasing evidences suggesting that TLR4 is also expressed in many tumor cell lines or tumors, especially epithelium-derived cancers[4]. In human head and neck squamous cell carcinoma[19], lung cancer[20], gastric carcinoma[21], ovarian tumors[22] and melanoma cells[23], TLR4 was all over-expressed, but not or lowly expressed in corresponding adjacent tissues or normal tissues. However, whether human pancreatic cancer cells express TLR4 remains to be understood.

In the present study, we found that not only TLR4 mRNA, but also TLR4 protein was over-expressed in PDAC compared with adjacent tissues. In addition, we found that the over-expression of TLR4 was related to tumor size, lymph node involvement, venous invasion and pathological stage. Kaplan-Meier analysis showed that the mean survival of patients without TLR4 expression in tumor tissues was significantly longer than those with TLR4 expression. These data indicated that TLR4 expressed in PDAC cells may contribute to its progression. Up to date, there have been increasing studies about effect and corresponding mechanism of TLR4 signal pathway on tumor progression. Pidgeon *et al*[24] found that endotoxin, a TLR4 agonist, introduced during surgery is associated with the enhanced growth of metastases following surgical trauma. In ovarian tumors, functional activity for TLR4 was demonstrated by stimulation of cell lines with specific ligands and subsequent activation and translocation of NF-κB and release of the proinflammatory cytokines interleukin-6 and CCL-2[22]. In human lung cancer cells[25], TLR4 ligation promoted production of immunosuppressive cytokines TGF-β, vascular endothelial growth factor (VEGF), proangiogenic chemokine IL-8, NF-κB was activated and contributed to apoptosis resistance of human lung cancer cells induced by LPS. Similar to other solid tumors, in pancreatic tumor microenviroment, there may also exist endogenous TLR4 ligands, which might be released during matrix degradation associated with tumor progression including HMGB1, heat shock proteins, hyaluronic acid and fibronectin fragment[22]. Many of these endogenous ligands may activate TLR4 signal pathway, induce inflammatory response, and promote tumor cells proliferation, apoptosis resistance, infiltration and metastasis. Thus, TLR4 might be a new marker of PDAC progression.

In solid tumors, cancer cells proliferation may outpace the rate of angiogenesis, result in tissue hypoxia, and cellular adaptation to hypoxia is a key step in tumor progression. This adaptation is regulated mainly by HIF-1 that is known to play an essential role in oxygen homeostasis[26]. In this study, we found the expression of both HIF-1α mRNA and protein was significantly higher in PDAC tissues than those in adjacent tissues, and the increased level of intracellular HIF-1α was associated with tumor size, lymph node involvement, venous invasion and pathological stage. These results were consistent with a previous study[27]. Wei *et al*[27] found that expression of HIF-1α was significantly higher in pancreatic cancer tissues, and there was significant difference in the expression of HIF-1α mRNA between Japanese Pancreatic Society stages Ⅰ-Ⅱ and stages Ⅲ-Ⅳ. Kaplan-Meier analysis also showed there was no significant

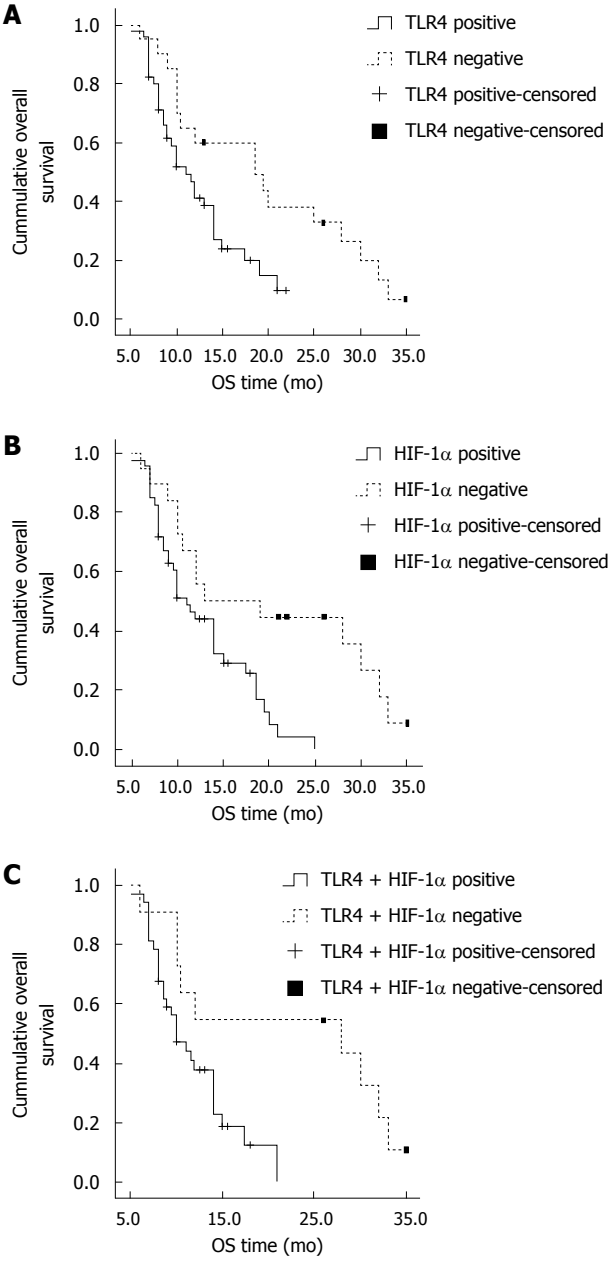


Figure 4 Kaplan-Meier analysis overall survival depending on TLR4 and HIF-1α expression in pancreatic ductal adenocarcinoma. A: The mean survival of patients with negative TLR4 expression in tumor tissues was significantly longer than those with TLR4 positive expression (P = 0.011); B: The mean survival of patients with negative HIF-1α expression in tumor tissues was significantly longer than those with HIF-1α positive expression (P = 0.005); C: Survival of patients with both negative TLR4 and HIF-1α expression was significantly longer than those with both positive TLR4 and HIF-1α expression (P = 0.014).

difference in the early stage of postoperation between negative and positive HIF-1α expression groups, but as time passed, the mean survival of patients without over-expression of HIF-1α in tumor tissues was significantly longer than those with over-expression of HIF-1α, which further demonstrated that patients with positive HIF-1α expression have worse prognosis. The main cause may be that upregulated and active HIF-1α forms complex with HIF-1β, translates into nuclear and activates the transcription of many genes that code for proteins involved in angiogenesis, glucose metabolism, cell proliferation, survival and invasion, metastasis[28]. Thus, the mortality of patients with over-expression of HIF-1α in the late stage increases because of recurrence, metastasis, chemotherapy and radiotherapy resistance. Thus, HIF-1α may serve as a useful molecular marker for prognosis of PDAC.

In the present study, we found that there was positive correlation between TLR4 and HIF-1α, indicating that there may exist a crosstalk between TLR4 signal pathway and HIF-1 signal pathway, which may act synergistically to promote the progression of PDAC. Recently, HIF-1α has also been shown to be up-regulated under normoxia in response to LPS[29]. In several reports, LPS, a ligand of TLR4, was found to induce accumulation and DNA-binding activity of HIF-1α protein in murine microglial cells[30], macrophage-differentiated cells[12], macrophages[31], and human myeloid monocytic leukaemia cells[32], which suggested that TLR4 signal pathway might regulate HIF-1α expression *via* some mechanism. van Uden *et al*[28] demonstrated that NF-κB was a direct modulator for HIF-1α expression. In our study, we also found that NF-κB p65 was over-expressed in PDAC. Considering NF-κB was an essential downstream component of TLR4 signal pathway[16], we speculated that the activation of TLR4 signal pathway in the microenviroment of PDCA might be partly involved in up-regulating HIF-1α *via* NF-κB pathway, then promoted progression of pancreatic adenocarcinoma.

In conclusion, the present study revealed that TLR4 and HIF-1α were over-expressed in pancreatic cancer, and both were related to the survival of patients with PDAC. On the other hand, there was positive association between TLR4 and HIF-1α. TLR4 signal pathway and HIF-1α signal pathway may synergistically promote development of the pancreatic cancer. However, the exact mechanism of crosstalk between HIF-1α and TLR4 in PDAC needs to be further studied.

COMMENTS

Background

Pancreatic ductal adenocarcinoma (PDAC) is a highly malignant digestive tumor with a very poor prognosis. Hypoxia-inducible transcription factor-1α (HIF-1α) is involved in malignant progression in many solid tumors, including PDAC, up-regulation of HIF-1α accelerates PDAC progression, but the exact regulatory mechanisms of HIF-1α in PDAC has not been unequivocally addressed. Recently, an increasing number of studies reported that toll-like receptors (TLRs) were upregulated in epithelial malignancies and involved in tumor progression, but whether TLRs, such as TLR4, is expressed on PDAC cells remains unknown. In immune- related cells, TLR signal pathway may induce expression of HIF-1α, but it is also still unclear whether there exists some association between TLR4 and HIF-1α in tumor microenviroment, such as PDAC.

Research frontiers

Activating TLRs pathway in tumor cells could promote the proliferation and inhibit the apoptosis, leading to migration, invasion and angiogenesis of tumor, but it remains unknown whether TLR4 is expressed and what role it plays in PDAC. In human cancers, HIF-1α is overexpressed as a result of intratumoral hypoxia. Recently, HIF-1α has also been shown to be up-regulated under normoxia in response to LPS, a potent agonist of TLR4, in immune-related cells, however, whether HIF-1α is regulated by TLR4 signal pathway in tumor is unknown. In this study, the authors demonstrate that TLR4 and HIF-1α are over-expressed in PDAC, and TLR4 expression is associated with HIF-1α. Nuclear factor-κB (NF-κB) p65, an important component in both TLR pathway and HIF-1 pathway, is also over-expressed in PDAC. The expression of TLR4 and HIF-1α has a

significant impact on survival of patients with PDAC. These results might indicate that TLR4 could be a potential factor for mediating HIF-1α in PDAC.

Innovations and breakthroughs

This is the first study to report that TLR4 is over-expressed in PDAC, and it is also the first report to show that TLR4 is associated with HIF-1α in PDAC.

Applications

The study demonstrated that TLR4, NF-κB p65 and HIF-1α were over-expressed in PDAC. The expression of TLR4 and HIF-1α was associated with tumor size, lymph node metastasis, venous invasion and pathological stage. Furthermore, the expression of TLR4 was positively correlated with expression of HIF-1α. Thus, TLR4 may be a novel marker for the progression and prognosis of PDAC, and may provide a new strategy for therapeutic intervention in the treatment of patients with PDAC in the future.

Peer review

This is an interesting study with valuable information regarding the expression and clinical correlation of TLR4 and HIF-1α in pancreatic ductal adenocarcinoma.

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BRIEF ARTICLE

Colchicine reduces procollagen Ⅲ and increases pseudocholinesterase in chronic liver disease

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Abstract

AIM: To test whether colchicine would be an effective antifibrotic agent for treatment of chronic liver diseases in patients who could not be treated with α-interferon.

METHODS: Seventy-four patients (46 males, 28 females) aged 40-66 years (mean 53 ± 13 years) participated in the study. The patients were affected by chronic liver diseases with cirrhosis which was proven histologically (n = 58); by chronic active hepatitis C (n = 4), chronic active hepatitis B (n = 2), and chronic persistent hepatitis C (n = 6). In the four patients lacking histology, cirrhosis was diagnosed from anamnesis, serum laboratory tests, esophageal varices and ascites. Patients were assigned to colchicine (1 mg/d) or standard treatment as control in a randomized, double-blind trial, and followed for 4.4 years with clinical and laboratory evaluation.

RESULTS: Survival at the end of the study was 94.6% in the colchicine group and 78.4% in the control group (P = 0.001). Serum N-terminal peptide of type Ⅲ procollagen levels fell from 34.0 to 18.3 ng/mL (P = 0.0001), and pseudocholinesterase levels rose from 4.900 to 5.610 mU/mL (P = 0.0001) in the colchicine group, while no significant change was seen in controls. Best results were obtained in patients with chronic hepatitis C and in alcoholic cirrhotics.

CONCLUSION: Colchicine is an effective and safe antifibrotic drug for long-term treatment of chronic liver disease in which fibrosis progresses towards cirrhosis.

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Key words: Chronic liver disease; Colchicine; Liver cirrhosis; Liver fibrosis; Type Ⅲ procollagen peptide

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INTRODUCTION

Hepatic fibrosis results from excessive deposition of extracellular matrix components, namely fibril-forming collagen type Ⅰ and Ⅲ. Fibrosis and cirrhosis develop after chronic injury from many causes, including persistent viral and helminthic infections, alcohol, autoimmune hepatitis, and metal overload. Early stages of fibrosis are reversible either by removal of the specific stimulus or by treatment with antifibrotic medications, whereas late stages, progressing to cirrhosis, are less reversible[1,2].

Our current knowledge of the pathology of hepatic

stellate cells (HSC), the main producers of type I collagen in the liver, as well as the molecular mechanisms involved in the activation of HSC and collagen production, have resulted in the discovery of new sites for potential therapeutic intervention. Among the various medical treatments, colchicine is a safe and efficient drug, based on our current knowledge of its pharmacodynamic[3-5], pharmacokinetic[6] and therapeutic[7-11] properties, and its ability to prevent or delay the development of hepatocellular carcinoma (HCC) in viral-related liver cirrhosis[12].

In this communication we present our findings after 4.4 years of follow up of a group of 74 patients who were randomly assigned to colchicine or control treatment. Our results show that colchicine treatment decreased mortality and significantly lowered serum levels of type Ⅲ procollagen peptide (P-Ⅲ-P) as compared with the control group.

MATERIALS AND METHODS

Patients

Seventy-four patients (46 males and 28 females) aged 40-66 years (mean 53 ± 13 years) participated in the study. Inclusion criteria were on an intention-to-treat basis. Exclusion criteria were: age < 20 years or a known hypersensitivity to colchicine. Patients were recruited by referral from general practitioner or by self choice and gave informed consent to be assigned to intervention (colchicine) or control by using random allocation. Randomization was performed by giving 74 consecutive numbers to all patients coming to our clinic between the years 2000 and 2003. Both the intervention group (group A) and the control group (group B) consisted of 37 patients. Group A was treated with colchicine (1 mg/d per os). Group B received the usual treatment for cirrhosis (diuretics, β-blockers, ursodeoxycholic acid, withdrawal of alcohol). Sixty-two patients were affected by different chronic liver diseases + cirrhosis (CLD + C), which was proven histologically in 58 of them (93.5%). In the four patients lacking histology, cirrhosis was diagnosed from serum laboratory tests, esophageal varices and ascites, consistent with clinical history. Twenty-eight patients (15 in group A, and 13 in group B) had post-hepatitis C cirrhosis (12 had been previously treated unsuccessfully with α-interferon for 12 mo), 20 patients (9 and 11 in each group, respectively) had post-hepatitis B cirrhosis, 10 (5 and 5) alcoholic cirrhosis, 4 (2 and 2) primary biliary cirrhosis. Among the 12 patients in whom cirrhosis was not found, 4 patients (2 and 2 in each group, respectively) had chronic active hepatitis C, 2 (1 and 1) chronic active hepatitis B, and 6 (3 and 3) chronic persistent hepatitis C (Table 1). Reasons for excluding the use of α-interferon for post-hepatitis C cirrhosis were: (1) previous unsuccessful treatment; (2) withdrawal owing to severe side effects; (3) autoimmune disorders; (4) severe depression; (5) thrombocytopenia; (6) decompensated cirrhosis; and (7) refusal. For these reasons the involvement of an Ethical Committee was thought not necessary. The study protocol conforms, however, to the ethical guidelines of the 1975 Declaration of Helsinki. The patients were followed up for a mean of 4.4 years, from 2000 to 2007, at the Centre for Metabolic Diseases and Atherosclerosis of Cagliari, Italy. During the study ten died (3 in group A and 7 in group B) and 12 (9 in group A and 3 in group B) withdrew from the study due to personal reasons.

Table 1 Diseases and number of patients participating in the study

Disease	Group A	Group B	*P*
Post-hepatitis C cirrhosis	15	13	0.65
Post-hepatitis B cirrhosis	9	11	0.65
Chronic active hepatitis C	2	2	0.70
Chronic active hepatitis B	1	1	0.85
Chronic persistent hepatitis C	3	3	0.85
Alcoholic cirrhosis	5	5	0.90
Primary biliary cirrhosis	2	2	0.80
Total	37	37	

Differences between the two groups were not significant statistically.

Serological tests

The liver function tests performed every year (half year initially) were: aminotransferases (mU/mL, normal values < 40), prothrombin time (%, normal values 70-100), pseudocholinesterase (mU/mL, normal values 5600-13 000), albumin (g/dL, normal values 3.5-5.0) and globulins (g/dL, normal values 0.6-1.0) displayed as an electrophoretogram. Serum N-terminal peptide of type Ⅲ procollagen (P-Ⅲ-P) was determined by radioimmunoassay, using the Cobra γ-counter device (ng/mL, normal values 4-14), as a marker of fibrogenesis in place of liver biopsy during follow-up[13].

Statistical analysis

Survival curves were plotted with the use of the Kaplan-Meier approach[14]. Differences in cumulative survival between the colchicine and the control groups were analyzed by means of the log-rank test[15]. Paired *t*-test of liver function tests between groups A and B, and in the same groups between baseline and 4-year values was performed. Ninety-five percent confidence intervals (95% CI) were calculated through interval estimation. Factor analysis for reducing individual scores from many to three was used[16] when analyzing the treatment outcome in patients with chronic liver disease C and in alcoholic cirrhosis using the following three criteria: serum levels of P-Ⅲ-P and γ globulins; serum pseudocholinesterase and albumin; subjective state of health.

RESULTS

Colchicine treatment was well tolerated, with transient diarrhea in a few cases. Overall, the best results obtained after 4 years of colchicine treatment were in subjects with chronic hepatitis C (20 cases, $P = 0.001$). In post-hepatitis B cirrhosis the results were less favourable and 2 of the 9 initial patients died before the 4th year. Nonetheless, in the remaining 7 patients a good result was obtained ($P = 0.05$ *vs* controls) (Table 2).

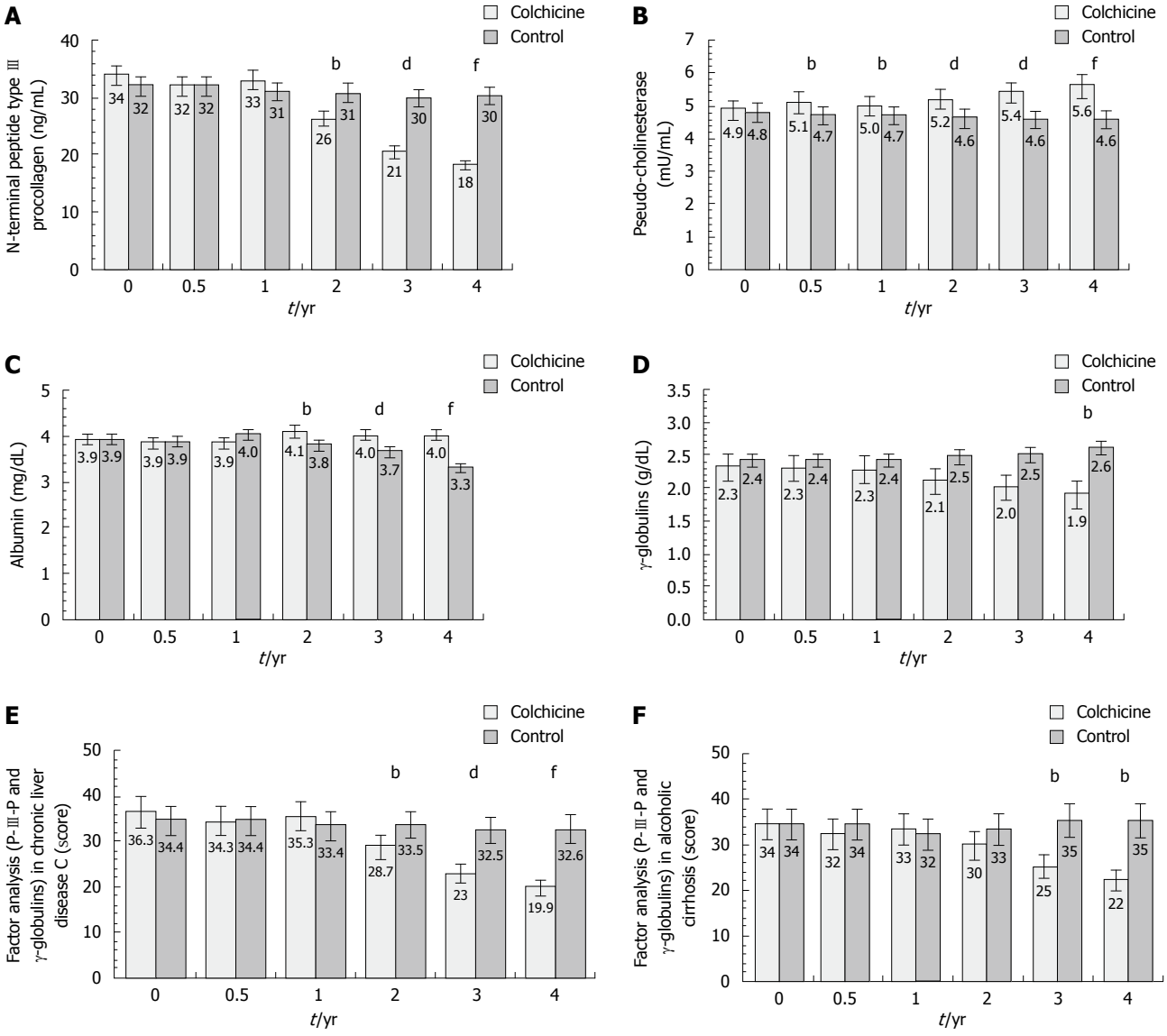


Figure 1 Type Ⅲ procollagen peptide (P-Ⅲ-P) (A), pseudo-CHE (B), albumin (C), γ-globulins (D), factor analysis (E, F) in colchicine and control groups. A-E: $^{b}P = 0.01$; $^{d}P = 0.001$; $^{f}P = 0.0001$; F: $^{b}P < 0.04$.

Table 2 Number of cases and number of improved patients for P-Ⅲ-P and pseudocholinesterase in the different liver disease groups treated for 4.4 years with colchicine

	Number of cases	Number of improved	*P vs* controls
Post-hepatitis C cirrhosis	15	13	0.001
Post-hepatitis B cirrhosis	9	11	0.05
Chronic active hepatitis C	2	2	0.001
Chronic active hepatitis B	1	1	0.50
Chronic persistent hepatitis C	3	3	0.001
Alcoholic cirrhosis	5	5	0.001
Primary biliary cirrhosis	2	2	0.55

P-Ⅲ-P: Type Ⅲ procollagen peptide.

Liver test values at baseline, 0.5, 1, 2, 3, and 4 years in the 37 patients treated with colchicine *vs* the 37 controls are reported in Table 3 and in Figure 1. In the colchicine group the most striking improvement was in serum P-Ⅲ-P values, which steadily declined from 34.0 at baseline to 26.2 at 2 years (-22.9%) and to 18.3 at 4 years (-46.2% from 34.0% to 18.3%, $P < 0.0001$, 95% CI: 19.4-17.1) (Table 3 and Figure 1A), and for pseudocholinesterase, the activity of which rose from 4900 at baseline to 5200 at 2 years (+6.1%) and to 5610 at 4 years (+14.5%, $P < 0.0001$, 95% CI: 5.7-5.5) (Table 3 and Figure 1B). These results are in striking contrast with the control group in which P-Ⅲ-P serum levels and pseudocholinesterase did not change significantly. Serum albumin and γ globulin levels also improved in the colchicine as compared with the control group ($P < 0.0001$, 4.1 to 3.9 and 2.2 to 1.5, respectively) (Figure 1C and D).

Survival of the 74 patients is reported in Table 4. Of the 37 patients in group A (colchicine), 36 survived at 2 years and 34 at 4 years. Of the 37 patients in group B (control), 35 survived at 2 years and 30 at 4 years. The survival figures showed a statistically significant benefit of colchicine ($P = 0.001$). The cumulative 4-year survival rate was 91.9% in group A and 81.1% in group B.

Table 3 Liver function tests at baseline and after 2 and 4 years

	Group A	%	*P*	Group B	%	*P*
P-Ⅲ-P baseline (ng/mL)	34.0			32.0		
P-Ⅲ-P 2 yr (ng/mL)	26.2	-22.9	0.01	30.8	-3.7	0.6
P-Ⅲ-P 4 yr (ng/mL)	18.3	-46.2	< 0.0001	30.2	-5.6	0.5
Pseudocholinesterase baseline (mU/mL)	4900			4800		
Pseudocholinesterase 2 yr (mU/mL)	5200	+6.1	0.05	4620	-3.7	0.6
Pseudocholinesterase 4 yr (mU/mL)	5610	+14.5	< 0.0001	4580	-4.6	0.6

Table 4 Survival of 37 patients treated with colchicines (Group A) and of 37 control patients (Group B) *n* (%)

	Group A	Group B	*P*
Baseline	37	37	
2 yr	36 (97.3)	35 (94.6)	0.05
4 yr	34 (91.9)	30 (81.1)	0.001

Table 5 Complications of cirrhosis in the two groups

Complications	Group A	Group B	*P*
Ascites	0	8	< 0.001
Encephalopathy	0	2	< 0.001
Esophageal hemorrhage	2	10	< 0.001

Group A: Colchicine, 20; Group B: Control, 18. Statistical significance of differences between the two groups: $P < 0.001$.

The complications of cirrhosis are reported in Table 5: ascites, encephalopathy and esophageal hemorrhage were almost absent in group A (2 esophageal hemorrhages *vs* 10 in group B, $P < 0.001$).

The factor analysis of serum P-Ⅲ-P and γ globulin levels in chronic hepatitis C showed a statistically significant improvement at 2, 3 and 4 years in the colchicine group (Figure 1E). In patients with alcoholic cirrhosis a significant change in the above parameters was reached at 3 and 4 years of colchicine treatment (Figure 1F). In contrast with these findings, a significant improvement in cholinesterase and albumin were observed until year 4 in chronic hepatitis C, while in alcoholic cirrhotic patients a statistically significant difference was found at 3 and 4 years of colchicine therapy. Regarding the overall subjective evaluation of colchicine patients with chronic hepatitis C and with alcoholic cirrhosis, a statistically significant difference, compared to controls, was found at 2, 3 and 4 years.

DISCUSSION

The striking improvement of CLD + C patients treated with colchicine, as compared with those on standard treatment, shows the effectiveness of colchicine for the treatment of hepatic fibrosis and cirrhosis[7-11]. Because this study was not designed to investigate a potential antiviral effect of colchicine, changes in viral DNA in patients with hepatitis B virus (HBV) were not investigated. Nonetheless, an antiviral effect of colchicine has been already documented by Floreani *et al*[9].

In the colchicine group, 9 patients stopped the treatment, *vs* only 3 patients in the control group, thus revealing a lower compliance with colchicine.

Multiple studies using colchicine for the treatment of chronic liver disease of different etiologies have shown variable results. In one study of hepatitis B patients, colchicine administered 5 mg/wk for 4 years showed a preventive effect towards evolution to cirrhosis (32.0% in treatment group A *vs* 73.2% in control group B), although of borderline significance[17].

In a study of hepatitis C, the combination of colchicine plus interferon resulted in worsening of alanine transaminase values and hepatitis C virus (HCV)-RNA levels compared to patients receiving only interferon[18].

In alcoholic cirrhosis treated with colchicine for up to 6 years, overall liver specific mortality was not reduced; however the alcoholic cirrhosis was advanced and, moreover, fewer patients on colchicine developed hepatorenal syndrome. The authors concluded that colchicine is not recommended for advanced cirrhosis[19], in agreement with Tome *et al*[20].

In primary biliary cirrhosis, where colchicine is indicated[4,7], the addition of colchicine to ursodeoxycholic acid was shown to result in a small but significant reduction in disease progression[21].

In addition to already cited papers[8,11], colchicine has been found to prevent the development of hepatocellular carcinoma in virally-related liver cirrhosis. However, the differences were not statistically significant when compared with a group receiving peginterferon (7.7% in interferon group and 5.9% in colchicine group ($P = 0.5$). The drug was less active than peginterferon alfa-2b in preventing variceal bleeding[22]. In 116 patients with virally-related liver cirrhosis on treatment with colchicine for a minimum of 3 years, hepatocellular carcinoma was prevented or delayed, compared with controls (9.0% *vs* 29.0%, $P = 0.01$), possibly through its anti-inflammatory effect[12].

In a study of 100 patients with various types of liver cirrhosis, colchicine was markedly better than control treatment as far as 10 years survival rates (56% *vs* 20%) and improvement of liver biopsies were concerned[23].

In another study in which the authors analyzed the combined results of 14 randomized clinical trials totalling 1138 patients, they concluded that colchicine should not be used for liver fibrosis or cirrhosis of various etiologies[24].

A study of the effect of colchicine combined with radiation showed that low doses of the drug possess radiosensitizing effects for HCC treatment[25].

Overall, our findings suggest that colchicine is anti-

fibrogenic based on the significant decrease in the levels of serum P-Ⅲ-P, a peptide derived from the processing of newly synthesized type Ⅲ collagen, an important component of liver scar tissue. This parameter has been used by others as a non-invasive procedure to follow liver fibrosis[13]. Thus, our findings together with the results obtained by Kershenobich *et al*[7] and other authors[8-11] clearly show that colchicine is effective for the treatment of some forms of liver cirrhosis. In this communication we report a significant improvement in patients with HCV who for various reasons declined to continue the use of interferon therapy. In the publication by Kershenobich *et al*[7] a significant improvement in patients with cryptogenic cirrhosis treated with colchicine was reported. Based on these findings it is possible to suggest that a better selection of patients, such as subjects with HCV who do not respond to interferon therapy, and early intervention could benefit a large population of patients for whom there is no alternative therapy.

In this study of patients with CLD + C, colchicine produced a clear-cut antifibrogenic effect, as indicated by the progressive decrease of serum P-Ⅲ-P and the parallel improvement of liver function tests.

Colchicine is an effective and safe antifibrotic drug for long-term treatment of chronic liver disease in which fibrosis progresses towards cirrhosis. It was shown not only to arrest, but even to reverse this process. With regard to the arrest of fibrosis, the stage of the disease at which colchicine treatment must be started is probably earlier than suggested at the present time. This does not mean that the drug provides an alternative to other well-established therapeutic strategies, but rather that colchicine should find its placement in association with the latter, except for combination with α-interferon in hepatitis C, where it displays unfavourable effects[18]. Removal of the initiating factor(s) is the best way, when feasible, to prevent the progression of hepatic disease[1,2]. With the addition of colchicine, this approach could be even more effective. Moreover, since liver cirrhosis is reversible[2,26], colchicine should be considered as a therapeutic agent. Histological evidence in addition to clinical non-invasive parameters could help in determining the antifibrogenic properties of colchicine. Accordingly, clinical trials of colchicine treatment in early stages of chronic liver disease are warranted.

COMMENTS

Background

Fibrosis and cirrhosis of the liver develop as a response to chronic injury from many causes. Early stages of fibrosis are reversible by treatment with antifibrotic drugs.

Research frontiers

Hepatic stellate cells provide a new site for potential therapeutic intervention with colchicine, which, besides its antifibrotic properties, can prevent or delay hepatocellular carcinoma.

Applications

There was a statistically significant survival benefit in the colchicine group, in which complications were almost absent. The plasma levels of P-Ⅲ-P declined progressively only in the colchicine group. The authors concluded that colchicine is an effective and safe antifibrotic drug.

Terminology

Fibrosis and cirrhosis develop after persistent viral and helminthic infections, alcohol abuse, autoimmune hepatitis or metal overload. Serum N-terminal peptide of type Ⅲ collagen (P-Ⅲ-P) is a marker of fibrogenesis, that can be exploited in place of liver biopsy. Plasma pseudocholinesterase and albumin can be measured as a response to P-Ⅲ-P.

Peer review

The article presents evidence for a drug, colchicine, which could prevent progression of liver disease.

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BRIEF ARTICLE

Endoscopic submucosal dissection training with pig models in a Western country

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Abstract

AIM: To test a strategy for endoscopic submucosal dissection (ESD) training in animal models designed to overcome the initial learning curve.

METHODS: ESD was attempted in *ex vivo* and *in vivo* pig models. Thirty ESD procedures were attempted in the esophagus (n = 9) or the stomach (n = 21). The *ex vivo* model was used until initial competence was achieved. In the *in vivo* model, several ESD procedures were performed in up to 3 sessions. The following variables were analyzed: specimen size, complete and *en bloc* resection rate, time for circumferential incision, time for submucosal dissection, total ESD duration, and complications.

RESULTS: Complete resection was achieved in 28 cases (*en bloc* 27); 2 could not be completed (one perforation, one technical difficulty). The mean ± SD time for circumferential incision was 36.2 ± 16.8 min (range: 8-87 min), and the mean ± SD time for submucosal dissection was 45.1 ± 35.7 min (range: 9-196 min). The mean ± SD size of the resected specimens was 45.2 ± 17.8 mm. The mean ± SD total resection time was significantly increased for the gastric cases performed in the first half of the study (n = 13) than in the second half (n = 8) (98.9 ± 62.4 min *vs* 61.7 ± 17.6 min, P = 0.04), although the specimen size did not differ.

CONCLUSION: Training in animal models could help endoscopists overcome the learning curve before starting ESD in humans.

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Key words: Endoscopic submucosal dissection; Training; Animal model

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Parra-Blanco A, Arnau MR, Nicolás-Pérez D, Gimeno-García AZ, González N, Díaz-Acosta JA, Jiménez A, Quintero E. Endoscopic submucosal dissection training with pig models in a Western country. *World J Gastroenterol* 2010; 16(23): 2895-2900 Available from: URL: http://www.wjgnet.com/1007-9327/full/

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INTRODUCTION

Endoscopic submucosal dissection (ESD) is an emerging technique for the treatment of early digestive neoplasms *en bloc*, which was developed in Japan and has been proven efficacious and safe in expert hands[1,2]. However, the applicability of ESD in Western countries is still unclear for several reasons. First, the most frequent indication for ESD is early gastric cancer, which is a common finding in Japan but rare in the Western world[3]. Second, ESD is a very operator-dependent technique, and a learning curve exists; it has been estimated that 30 gastric cases need to performed under the supervision of an expert to overcome this learning curve[4,5]. For endoscopists at the beginning of the learning curve, the perforation rate can reach 20%, especially in the colon. Third, colorectal ESD could probably become a frequent indication for ESD in Western countries, but that is the most difficult location in which to perform ESD[6]. Although experts have suggested that ESD should be carried out in pigs before starting to apply it in humans - especially when it is performed without the supervision of an expert - there is no full published study reporting the results of ESD in pigs as a part of the training for this difficult therapeutic procedure[5]. Our hypothesis is that the strategy of learning ESD from experts, followed by training in harvested pig stomachs and then in live pigs, may be useful for overcoming the learning curve. The aim of our study was to prospectively determine the results, efficacy, and safety of ESD performed in pigs by an endoscopist at the beginning of the learning curve prior to its application in humans.

MATERIALS AND METHODS

Prior learning

One author (Parra-Blanco A) spent 1 mo (November 2006) at the National Cancer Center Hospital (NCCH), Tokyo, where he observed approximately 25 cases of ESD (esophageal, gastric, and colonic). He also performed 7 gastric ESD procedures in harvested stomachs under the supervision of an expert. The results of these procedures were not recorded, but all of them were gastric resections with an approximate greater diameter of the resected specimen of 3 cm.

Animal models

***Ex vivo* model:** A model for training in isolated esophaguses and stomachs, similar to the one previously used at the NCCH, was prepared at the first author's institution. A plastic box (40 cm × 25 cm × 15 cm) was used that had a 15 mm in diameter hole in one of the short sides. Stomachs with the whole esophagus attached were obtained from female, 70 kg, domestic pigs. They were washed vigorously with lukewarm water and a mucolytic (10% N-acetyl-cysteine). The stomach was laid on a metal plate inside the plastic box. An overtube was introduced into the hole and then inserted into the esophagus. Plastic clamps were employed to tighten the esophagus to the overtube and prevent it from sliding with the movements of the endoscope.

A dedicated endoscope was used (Fujinon EG-200HR) for all procedures in this study.

***In vivo* model:** Live, *Sus scrofa,* female, 30 kg, domestic pigs were used. The pigs fasted for 48 h prior to the procedure, and clear liquids were allowed *ad libitum*. Intramuscular (im) ketamine (11 mg/kg) was administered as a premedication. Induction was accomplished with intravenous (iv) propofol (16 mg/kg) and inhalation anesthesia with isoflurane.

When the procedure was finished, im buprenorphine (0.01-0.04 mg/kg) and iv omeprazole (40 mg) were administered. After ESD, the pigs only had clear liquids for 24 h, hypercaloric enteral nutrition solution for the next 24 h, and then fodder. Esomeprazole (40 mg) *per os* was given daily until the sacrifice of the pigs.

Each pig could be subjected to general anesthesia a maximum of 3 times, with an interval of 2 wk between sessions. One to 3 ESD procedures were performed during each session at the endoscopist's will, depending on the duration of the procedures. The pigs were monitored by the veterinary staff for signs of sepsis, distress, pain, and changes in feeding habits or behavior. The animals were sacrificed either at the end of the third session or at any time before that in the event of complications that could not be easily managed endoscopically.

The study was approved by the Animal Study Committee of our university.

Endoscopic procedures

All endoscopic procedures were carried out by a single experienced endoscopist (Parra-Blanco A). During each session, both with the harvested stomach and the live pig, he performed as many ESD procedures as desired, although a maximum limit of 5 h for the whole session was set. The ESD technique applied was basically similar to the one generally performed at the NCCH. ESD was attempted in the gastric body or the antrum of the stomach, excluding the fundus and the subcardial area. In the esophagus, generally the middle portion was selected, avoiding the esophago-gastric junction.

A needle knife (Olympus, KD 10Q-1) was usually used for the marking of the periphery of the selected area and the initial cut. After submucosal injection of fluid (10% glycerin) to raise the submucosal layer, at least 2 cuts were made usually at the 12 and 6 o'clock positions on the endoscopic image 5-10 mm away from the marking line. The insulated-tip (IT) knife (Olympus KD-610L, Tokyo, Japan) was generally used for the circumferential cutting of the mucosa surrounding the lesion and the dissection of the submucosal layer under the lesion. However the endoscopist could switch to any of the other devices: needle knife, Flex knife (Olympus KD-630L, Tokyo, Japan), and Hook knife (Olympus

KD-620 LR, Tokyo, Japan). For the dissection phase, a transparent hood was used (Olympus D-201-12704, Tokyo, Japan). Snaring of the partially dissected mucosa was considered only as the last choice in cases in which it was not possible to resect the lesion *en bloc* with the other devices. The Erbotom ICC 200 ERBE system was used (ERBE, Elektromedizin GmbH, Tübingen, Germany) as a high-frequency generator.

In both models, the resected specimens were pinned on a cork and then measured.

The following variables were prospectively recorded: (1) location of the targeted area (esophagus *vs* stomach); (2) endoscopic accessories employed; (3) time required from the marking until the end of the circumferential incision (circumferential cutting); (4) time from the end of the circumferential cutting until the end of the dissection (submucosal dissection); (5) size of the resected specimen; (6) completeness of the resection (complete *en bloc*, complete piecemeal, incomplete); and (7) complications (immediate bleeding, delayed bleeding that occurred after completion of the ESD, open perforation, and nontransmural damage to the proper muscle layer when electrocoagulation produced a distinct depression in that layer but without any open perforation).

Statistical analysis

A global χ^2 test was used to compare qualitative data. Continuous variables were compared with the nonparametric Mann-Whitney *U* test, and mean ± SD are reported. Calculated *P* values < 0.05 were considered to indicate statistical significance.

RESULTS

Thirty ESD procedures were carried out, 8 in the *ex vivo* model (esophagus 2, stomach 6) in 6 harvested organs and 22 in the *in vivo* model (esophagus 7, stomach 15) in 6 live pigs, during a 51 wk period (0.57 ESD/wk). Specific technical aspects corresponding to the 30 cases, including the types of knives employed, are presented in Table 1.

The mean ± SD time to perform the circumferential incision was 36.2 ± 16.8 min (range: 8-87 min), and the mean ± SD time to perform the submucosal dissection was 45.1 ± 35.7 min (range: 9-196 min). One procedure had to be stopped before completion because of a technical difficulty (case 20). This and another case in which a large perforation occurred were the only 2 cases of incomplete resection. Therefore, the resection was complete in 28/30 (93.3%). In 2 cases, the dissection had to be completed with a snare; this resulted in a piecemeal resection in one of these cases (case 12). Therefore *en bloc* resection was achieved in 90% (27/30) cases. Prophylactic hemostasis was conducted on the post-resection ulcer in 16/22 (72.7%) cases in the live pig model (with the IT knife or Flex knife). The mean ± SD size of the resected specimens was 45.2 ± 17.8 mm (22-116 mm). Table 2 presents results of the ESD procedures performed in the *ex vivo* and *in vivo* models.

When the first half of the cases was compared with the second half, no significant difference in the duration of the procedures was observed (93.1 ± 59.9 min *vs* 70.1 ± 24.6 min, $P = 0.34$). However, when only the gastric cases were considered, the mean ± SD time required from the end of the circumferential cutting until the end of the dissection was significantly longer in the gastric cases performed in the first half of the study ($n = 13$) than in the second half ($n = 8$) (58.6 ± 47.8 min *vs* 25.2 ± 11.4 min, $P = 0.025$). The mean ± SD total time for the resection was also significantly increased in the initial 13 gastric cases compared to the last 8 cases (98.9 ± 62.4 min *vs* 61.7 ± 17.6 min, $P = 0.045$). However, the size of the resected gastric specimens did not differ between cases performed during the first *vs* the second half of the study (49.3 ± 23.1 mm *vs* 50.1 ± 10.7 mm, respectively, $P = 0.34$).

Comparison of the gastric cases revealed that the mean total procedure times for the 6 *ex vivo* cases, the first 8 *in vivo* cases, and the last 7 *in vivo* cases were not significantly different. The mean sizes of the resected specimens also did not differ among the groups (Table 2).

In the *ex vivo* model, the locations of the resected areas were the lesser curvature ($n = 4$) and the greater curvature ($n = 2$). In the first 8 *in vivo* cases, the locations of the resected areas were the lesser curvature ($n = 1$), greater curvature ($n = 6$), and anterior wall ($n = 1$), and in the last 7 *in vivo* cases, the locations were the lesser curvature ($n = 2$), greater curvature ($n = 3$), and anterior wall ($n = 2$).

Comparison of the esophageal cases revealed that the mean total procedure times for the 2 *ex vivo* cases, the initial 3 *in vivo* cases, and the last 4 *in vivo* cases were 55.0 ± 7.1 min, 98.7 ± 23.2 min, and 65.5 ± 26.5 min, respectively, without significant differences among the groups (Table 2).

Complications

In 5/22 (22.7%) cases there was bleeding requiring coagulation with hot biopsy forceps, and all were easily managed without hemodynamic instability. There was nontransmural damage to the muscular layer in 4/9 (44.4%) esophageal cases and in 3/21 (14.3%) gastric cases, which was generally closed with clips. In the fourteenth case, a large upper gastric perforation occurred when the Hook knife was being used, requiring animal sacrifice. The only complication observed during follow-up was one case of esophageal stricture, which was easily dilated endoscopically.

One pig died 1 wk after undergoing gastric and esophageal ESD procedures; a necropsy showed signs consistent with cardiac tamponade, apparently unrelated to the endoscopic resections.

DISCUSSION

ESD is an emerging technique that allows for safe and effective *en bloc* resection of gastrointestinal neoplasms in expert hands[7]. However, it is technically demanding and, therefore, very operator-dependent. In Japan and some other Asian countries, training in this technique relies on supervision by experts, who take over the examination

Table 1 Technical details of the 30 ESD procedures

	Model type	Organ	Type of knife	Specimen size (mm)	Procedure duration (min)		Immediate complications
					CC	D	
1	EV	Stomach	NN + IT	31	28	46	-
2	EV	Stomach	NN + IT	35	23	28	-
3	EV	Esophagus	NN	28	15	35	-
4	EV	Stomach	NN + IT	35	56	33	-
5	EV	Esophagus	NN + IT	31	44	16	NDML
6	EV	Stomach	FN	38	35	23	-
7	EV	Stomach	FN + IT	48	25	60	NDML
8	EV	Stomach	FN + HN	49	35	45	NDML
9	IV	Stomach	FN + IT	48	25	80	-
10	IV	Stomach	NN + IT	67	15	30	NDML
11	IV	Stomach	NN + IT	41	57	23	-
12	IV	Stomach	NN + IT + S	37	38	24	Bleed
13	IV	Stomach	NN + IT	116	60	83	Bleed
14	IV	Stomach	NN + FN + HN + IT	-[1]	87	196	Perforation
15	IV	Stomach	NN + IT	45	40	91	Bleed
16	IV	Esophagus	NN + FN + HN + IT	45	39	42	-
17	IV	Esophagus	FN + IT	50	55	70	NDML
18	IV	Stomach	NN + IT	45	28	9	-
19	IV	Esophagus	FN + IT	53	42	48	Bleed
20	IV	Stomach	NN + IT	-[1]	47	26	-
21	IV	Stomach	NN + IT	54	43	27	-
22	IV	Stomach	NN + IT	62	49	27	-
23	IV	Esophagus	NN + FN + HN + IT	32	28	27	-
24	IV	Stomach	NN + IT	62	45	16	-
25	IV	Stomach	NN + IT	45	20	17	-
26	IV	Esophagus	NN + FN + IT	22	8	25	-
27	IV	Esophagus	NN + IT + S	35	17	73	NDML
28	IV	Stomach	FN + IT	51	35	45	Bleed
29	IV	Esophagus	FN + IT	30	32	52	NDML
30	IV	Stomach	NN + IT	32	15	35	-

[1]Resection was not completed, and therefore, no specimen was pinned or measured. ESD: Endoscopic submucosal dissection; EV: *Ex vivo*; IV: *In vivo*; NN: Needle knife; IT: Insulated-tip knife; HN: Hook knife; FN: Flex knife; S: Snare; CC: Time from marking until circumferential cutting; D: Time from circumferential cutting until end of dissection; Bleed: Bleeding requiring hot forceps coagulation; NDML: Non-transmural damage of the proper muscular layer.

Table 2 Results of the 30 ESD procedures performed in the *ex vivo* model, *in vivo* model 1 (first 11 resections in the live pigs), *in vivo* model 2 (last 11 resections in the live pigs) (mean ± SD)

	n	Size (mm)	Time from marking until circumferential cutting (min)	Time from circumferential cutting until end of dissection (min)	Total time (min)	Proper muscle layer injury (*n*)	Open perforation (*n*)	Major bleeding (*n*)
Esophagus								
Ex vivo	2	29.5 ± 2.1	29.5 ± 20.5	25.5 ± 13.4	55.0 ± 7.1	1	0	0
In vivo 1	3	49.3 ± 4.0	45.3 ± 8.1	53.3 ± 14.7	98.7 ± 23.2	1	0	0
In vivo 2	4	29.7 ± 7.6	21.2 ± 10.9	44.2 ± 22.8	65.5 ± 26.5	2	0	0
Stomach								
Ex vivo	6	45.3 ± 8.5	33.7 ± 12.0	39.2 ± 13.7	72.8 ± 15.2	2	0	0
In vivo 1	8	57.0 ± 27.7	43.7 ± 23.3	67.0 ± 61.1	110.7 ± 79.5	1	1	0
In vivo 2	7	51.0 ±11.3	36.3 ± 13.6	27.6 ± 10.0	65.3 ± 15.7	0	0	0

when the trainees face technical difficulties or when serious complications (e.g. perforation) occur[5,8]. Because the technique is seldom practiced in Western countries, the best training strategy and the applicability of ESD remain unclear. Dinis-Ribeiro *et al*[9] have published results from an initial series of gastric ESD procedures performed in humans in a Western country. The ESD procedures in that study were performed by a single endoscopist who was trained in Japan, and animal training preceded application to human cases. However, the authors did not specifically describe the nature of the animal cases performed. Probst *et al*[10] reported 71 epithelial or submucosal lesions treated by ESD in a single German institution. They showed good results, with open gastric perforations in only 2 cases. The authors found a significant reduction in the procedural time in the second half of the cases; however this improvement might be also related to the training by a Japanese expert at that time. No previous animal training was described in this study.

In this study, we proposed a strategy for training in ESD in the absence of experts to supervise the procedures and ensure the patients' safety. In some previous

studies, pig models have been used to investigate new methods for ESD[11-13]. A European study evaluated the usefulness of a new endoscope with a double channel[14]. Six expert endoscopists participated in this study, in which 17 ESD procedures were performed in live pigs and 10 in humans during the clinical part of the study. The mean number of resections per endoscopist in this study was less than 5 - less than the estimated 30 cases of ESD needed to overcome the learning curve - and the effect of learning was not assessed. Therefore, to our knowledge, ours is the first study to evaluate the usefulness of pig models for ESD training, including a significant number of cases. Vázquez-Sequeiros *et al*[15] recently described the use of porcine models similar to those employed in this study, prior to attempting a human gastric ESD which was successful.

Choi *et al*[4] showed that when prior knowledge of advanced resection techniques is limited and no supervision by an expert in ESD is available, there is a learning curve in which not only the *en bloc* resection rate and procedure duration improve with increasing experience but, more importantly, the perforation rate decreases too. However, the technique employed in that study was not really what is currently understood as ESD, because a snare was used to encircle the lesion after margin cutting. Therefore, it is likely that if the real ESD technique is applied without any previous experience or supervision by experts, the complication rate would be greater than what was reported by Choi *et al*[4]. Therefore, self-taught learning is not desirable[6]. On the contrary, there do not seem to be any differences in the perforation rates between trainees and experts when the former are supervised by the latter[8]. Hence, training in animal models may not be needed in Japanese institutions where supervision by experts is available.

The incidence of gastric cancer is higher in Japan compared to that in most Western countries, where the number of early gastric lesions detected is low[16]. In those countries, it could be expected that colorectal and esophageal lesions (mainly Barrett's dysplasia) could be a frequent therapeutic indication for ESD. Performing ESD in such locations is especially complex and technically demanding, with an increased perforation risk[7]. Training in animal models appears to be a compulsory step before attempting to perform ESD in the absence of experts, which is the current situation in Western countries. Otherwise, it can be expected that the development of ESD outside of Japan and neighbouring countries will be very limited. Besides, prior training in animal models may carry medico-legal implications, and such previous experience could support the idea that the endoscopist is fit to perform ESD.

Our results suggest that there exists a learning curve for ESD in animal models. In the gastric cases, the time required for dissection was significantly reduced in the second half of the study, although the sizes of the resected specimens were similar. A large perforation occurred in one of the live pigs in our study, for which suture by clipping was considered impossible. Had such a large perforation occurred in a real patient, emergent surgery would certainly have been required.

In our strategy, we included previous learning from world experts in ESD, although the effect of that variable was not been tested in our study. Training in pigs could be started without such previous learning. However, there are certain technical tricks and tips that are difficult to learn by oneself, and ESD involves manoeuvres that traditionally have not been used during flexible endoscopy, such as the lateral cutting. Thus, to achieve the ability to perform ESD safely in humans, we believe that learning from experts is a must. Other possible strategies would be to organize training courses (with animal and/or human training) under the supervision of experts, or to attempt ESD procedures supervised by means of a videoconference. We have carried out both actions in our department, but its impact on ESD performance has yet to be reported.

The main limitation of our study may be the fact that the effect of training was assessed only in animal models; thus, there is no description of results from when the technique is applied in humans by the same endoscopist. The endoscopist who performed the cases in the present study has performed thereafter 6 cases in humans (5 gastric, 1 rectum) without any serious complication. A greater number of cases in humans has to be performed and the results monitored in order to determine the usefulness of ESD training with the strategy described in this study.

Although the endoscopic manoeuvres are certainly similar, regardless of whether a human or a pig is being endoscoped, there are several differences between the pig and human stomach that may contribute to technical differences. Firstly, it is accepted that bleeding in the pig is more infrequent and subtle. Secondly, the pig's mucosal layer appears to be thicker, as suggested by the fact that the submucosal injection frequently becomes very difficult to accomplish. Thirdly, as normal mucosa is resected in the pig model, no significant fibrosis is expected to be encountered during pig ESD. In contrast, fibrosis is a relatively frequent finding during ESD in humans, which makes the procedure more challenging. Fourthly, *ex vivo* and *in vivo* models were analyzed together in our study, and there might be certain peculiarities related to the 2 different models that could have influenced our results. However, our *ex vivo* model was prepared just a few hours after the animal was sacrificed. This is in contrast to some *ex vivo* models that are prepared after having kept the stomach frozen, which makes the tissue harder and probably more difficult to resect. Moreover, the sizes of the organs in the *ex vivo* models were quite larger than those in the *in vivo* models, as the former organs were obtained from 70 kg pigs and the latter, from 30 kg pigs. It was our impression that in the cases involving large *ex vivo* stomachs (which more closely resembled real human stomach), it was much easier to approach the lesser curvature. However, in the cases involving small *in vivo* stomachs, the lesser curvature was short and difficult to approach adequately with the gastroscope. For that reason, more areas in the greater curvature were targeted in the *in vivo* cases.

In order to fully understand the usefulness of *ex vivo* and *in vivo* models, future studies should compare the results of ESD performed in similarly sized stomachs in *ex vivo* and *in vivo* models.

Lastly, 7 gastric ESD procedures performed in the *ex vivo* model by the author supervised by the Japanese experts were not included in this study, as they were not videotaped or the procedure times registered. As they were done at the beginning of the learning curve, they were especially time consuming. Had those cases been included, the evidence of a learning curve might have been stronger.

In conclusion, ESD is a technically challenging procedure and there seems to be a learning curve, as suggested by this study. As potentially serious complications can occur in the absence of local expertise, it should probably not be applied to humans by endoscopists without any previous animal training. The learning strategy described provided good results in terms of efficacy and safety, as a prior step to its application in humans.

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COMMENTS

Background

Endoscopic submucosal dissection (ESD) allows for *en bloc* resection of early neoplasms in the gastrointestinal tract. However, it is associated with a high rate of perforations, and it should be learnt from experts. In the absence of experts in western countries, practising in animal models may be helpful to overcome the learning curve.

Research frontiers

The results of large series of ESD in humans after previous training with animal models have to be clarified.

Innovations and breakthroughs

In this study, a strategy was proposed for ESD training, which included visiting an expert center, and then practicing gastric and esophageal ESD on *ex vivo* and finally on *in vivo* porcine model. The results of these ESDs were analyzed. The number of cases included was 30, as this is considered to represent the workload required to overcome the initial learning curve.

Applications

Complete resection was achieved in 93% cases. The dissection phase was the most time consuming, and as suggested by other studies this seems to be the most challenging for beginners. There seems to be a learning curve for gastric ESD when this training model is applied; therefore it would be justified to start training on animal models instead of on real patients, if experts are not available to supervise the procedures.

Terminology

Endoscopic submucosal dissection: this is an endoscopic method for complete *en bloc* resection of early gastrointestinal neoplasms, whose major drawback is that it is technically challenging and associated with a significant risk of complications mainly during the learning curve.

Peer review

It is a useful, structured experiment to demonstrate the effectiveness of using a pig model for ESD training. It showed technical skill of ESD could be improved by practice in the pig. It provides an alternative way to learn ESD in the future.

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BRIEF ARTICLE

High-sensitivity C-reactive protein in paediatric inflammatory bowel disease

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Abstract

AIM: To study whether high-sensitivity C-reactive protein (hs-CRP) measurement can aid the assessment of disease activity and glucocorticoid treatment in paediatric inflammatory bowel disease (IBD).

METHODS: CRP levels were measured in 39 children with IBD undergoing colonoscopy [median age 12.8 years, Crohn's disease (CD) n = 20], in 22 other children with IBD followed for acute response to glucocorticoids, and in 33 paediatric non-IBD patients. When standard CRP level was below detection limit (< 5 mg/L), hs-CRP was analyzed.

RESULTS: Sixty-four percent (25/39) of the children with IBD undergoing colonoscopy displayed undetectable (< 5 mg/L) standard CRP levels. Of these, the hs-CRP measurement could not differentiate between active (median, 0.2 mg/L, range, 0.007-1.37, n = 17) or quiescent (0.1 mg/L, 0.01-1.89, n = 8, P = NS) disease. Patients with ileocolonic CD had higher CRP levels (14 mg/L, 0.06-45, n = 13) than patients with no ileal involvement (0.18 mg/L, 0.01-9, n = 7, P < 0.01) or ulcerative colitis (UC) (0.13 mg/L, 0.007-23, P < 0.05). In children with active IBD treated with systemic glucocorticoids, the standard CRP was undetectable in 59% of the patients. The hs-CRP levels did not differ between patients that responded to steroid therapy and in non-responders.

CONCLUSION: The measurement of hs-CRP did not prove useful in the assessment of disease activity or glucocorticoid treatment in paediatric IBD patients that had undetectable standard CRP.

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Key words: C-reactive protein; Crohn's disease; Ulcerative colitis; Children; Inflammatory markers

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INTRODUCTION

C-reactive protein (CRP) is produced mainly in hepatocytes in response to acute phase stimuli such as inflammation[1]. Its production is driven by circulating cytokines interleukin-6 (IL-6), IL-1β and tumor necrosis factor-α (TNF-α) originating from the inflamed tissues[2]. Genetic

variation, as well as age and sex affect the CRP levels[3]. As an acute phase response, CRP concentration may increase rapidly up to 1000-fold[4].

CRP is commonly used to screen the activity of chronic inflammatory diseases including inflammatory bowel disease (IBD). A study comparing CRP with clinical, endoscopic and radiologic findings in adults with IBD found an association between CRP, clinical disease activity, endoscopic inflammation and active histological inflammation[5]. Very recently however, a Norwegian group reported that only 29% of adult patients with ulcerative colitis (UC) and 75% of patients with Crohn's disease (CD) had elevated CRP levels at diagnosis[6]. In general, CD patients have higher CRP levels than patients with UC[7]. Still, also in CD a subgroup of patients with clinically active disease present with low CRP. In two large clinical studies investigating new biological agents (CDP870 and natalizumab, respectively) in active CD, 60% of the patients had CRP levels below 10 mg/L and 25% below 3 mg/L[8,9].

In children, the data on CRP levels in active IBD is sparse. In a report from St Bartholomew's Hospital, all paediatric patients with active CD (n = 26) had CRP values higher than 5 mg/L; of patients with UC (n = 13), only 60% reached this cut-off level[10].

High-sensitivity CRP (hs-CRP) assays measure CRP levels that were previously thought to be under the detection limit. In adult IBD patients, hs-CRP correlates with clinical disease activity in both UC and CD[11]. In children, this question has not been addressed. Therefore, in this pilot study we investigated the association between hs-CRP and clinical and histological activity in paediatric IBD patients, and evaluated the effect of glucocorticoid treatment on the hs-CRP levels.

Table 1 The background data of the paediatric IBD patients with CRP measurement at the time of the colonoscopy (n = 39)

UC	19
M/F	10/9
Pancolitis	16
Left-sided disease	3
CD	20
M/F	14/6
Ileocolitis	13
Colitis	7
Age (median, range, yr)	12.8 (1.9-18)
BMI (median, range, kg/m^2)	18.40 (14.2-25)
Fresh diagnosis	20/39

Notably, all patients with Crohn's disease presented with colitis. IBD: Inflammatory bowel disease; CRP: C-reactive protein; UC: Ulcerative colitis; CD: Crohn's disease; BMI: Body mass index.

Table 2 The clinical characteristics of the 22 paediatric IBD patients followed for acute response to glucocorticoids

UC	14
M/F	9/5
Pancolitis	11
Left-sided disease	3
CD	7
M/F	5/2
Ileocolitis	2
Colitis	5
IC	1 male
Age (median, range, yr)	13.1 (3.5-18)
BMI (median, range, kg/m^2)	16.9 (13.2-22.6)
Fresh diagnosis	7/22

IC: Indeterminate colitis.

MATERIALS AND METHODS

Subjects

Study group Ⅰ: Paediatric IBD patients studied at the time of colonoscopy: The first group was stratified from the consecutive paediatric IBD patients who underwent colonoscopies and agreed to participate in a follow-up study of IBD patients at the Hospital for Children and Adolescents, Helsinki, Finland. Inclusion criteria to the study were (1) endoscopy-based diagnosis of either UC or CD[12], and (2) age ⩽ 18 years. Exclusion criteria were (1) previous surgery, and (2) any signs of infection during the preceding week.

The clinical characteristics of the 39 patients are shown in Table 1. 20 patients were newly diagnosed. The other 19 patients received the following medication: 7 patients were on 5-ASA, 3 patients on 5-ASA and glucocorticoid, 3 patients were on 5-ASA, glucocorticoid and azathioprine, 1 patient on 5-ASA and antibiotics, 1 patient on glucocorticoid and azathioprine, and 1 patient on glucocorticoid, azathioprine, antibiotics and infliximab. Two patients had no medication due to quiescent disease and 1 patient had no medication due to non-compliance.

The patients who underwent colonoscopy due to fresh disease, aggravation of the symptoms or lack of response to conventional medication were defined as having clinically active disease according to Physicians Global Assessment (PGA). The patients who underwent colonoscopy in clinical remission according to PGA were categorized as having quiescent disease.

Study group Ⅱ: Paediatric IBD patients followed up for acute response to glucocorticoids: The second group was a prospectively collected group of 22 children and adolescents (median age 13.1 years, age range 3.5-18 years) with active IBD who were introduced to systemic glucocorticoid treatment due to active disease (Table 2) as described in detail[13]. Six patients in this study were recruited to the prospective extension of the previous study[13]. Other inclusion criteria to the study were similar to group Ⅰ. The maintenance medication of the patients at the onset of the steroid was: 5-ASA (n = 12), 5-ASA and azathioprine (n = 2), antibiotics (n = 4), no medication (n = 4).

The glucocorticoid treatment was started either with peroral prednisolone (1 mg/kg per day, Prednisolone®, Leiras, Finland, n = 20) or budesonide (9 mg/d, Entocort®, AstraZeneca AB, Sweden, n = 2). The venous blood samples were drawn before the treatment was started and at the clinical follow-up visit at 2-4 wk after starting the glucocorticoid.

Clinician's judgement (PGA) of clinical improvement (decrease in stool rate and the amount of blood in the stools, abating diarrhea) and decrease in the inflammatory markers [erythrocyte sedimentation rate (ESR) and faecal calprotectin] by the time of the follow-up visit (see above) was defined as therapeutic response. When these patients were recruited, no paediatric activity index for UC yet existed[14]. Acute (appearing during the first month of the treatment) glucocorticoid-related side effects (weight gain, development of moon face, striae or acne) were registered[13]. The therapeutic response and the development of side-effects were both graded with a two-grade scale (good/poor response, yes/no side effects)[15,16].

Controls: Thirty-three children and adolescents (median age 12.1 years, range 1.8-17.2 years) visiting the Outpatient Clinic of the Hospital for Children and Adolescents, Helsinki, Finland, due to gastrointestinal disorder (coeliac disease $n = 5$, loose stools $n = 4$, liver dysfunction $n = 3$, pancreatic dysfunction $n = 3$, abdominal pain $n = 2$, Helicobacter gastritis $n = 1$, gastro-oesophageal reflux disease $n = 1$), growth follow-up $n = 4$, derangement in laboratory tests (hypercholesterolemia $n = 3$, leukopenia $n = 1$, sideropenia $n = 1$) or other reasons (obesity $n = 1$, fatigue $n = 2$, talassemia $n = 1$, and toe pain $n = 1$) served as non-IBD controls. Patients with acute infection, fever or trauma were excluded.

Histology

During the colonoscopies, routine biopsies were taken from the ileum, caecum, colon ascendens, transversum, descendens, sigma and rectum. The histological findings were graded by an experienced paediatric pathologist (R.K.) with a modified histology score originally developed for CD (Table 3)[17,18]. The score was calculated: (1) separately for each segment (max 15 points) and (2) adding the scores of the caecum, colon ascendens, transversum, descendens, sigma and rectum to obtain the total score for the colon (max 90 points). Eosinophils were taken into account when analysing the quantity of the polymorphonuclear cells. Instead, lymphoid hyperplasia was omitted from the count, since it represents a normal variant. The presence of granulomas was registered, but not calculated in the score.

Laboratory analyses

The venous blood samples were scheduled between 11 am and 3 pm. Blood count, standard CRP (detection limit 5 mg/L), and ESR were analysed in a clinical laboratory. In patients with standard CRP levels under the detection limit, serum hs-CRP levels were measured. The hs-CRP analyses were performed with a human C-reactive protein Instant ELISA kit (Bender MedSystems GmbH, Vienna, Austria) that has an intra-assay coefficient of variation (C.V.) of 6.9%, interassay C.V. of 13.1% and detection limit of 3×10^{-6} mg/L. Faecal calprotectin was quantified as previously reported[19].

Statistical analysis

Mann-Whitney's *U* test, Kruskal-Wallis test, Wilcoxon's signed rank sum test, Fisher's exact test and Spearman's rank order correlation test were used when investigating associations between non-parametric variables. Statistical analyses were performed using SPSS by SPSS Inc. software. $P < 0.05$ was accepted to indicate statistical significance. Values are expressed as median (range) in the text.

Table 3 The assessment of the histology score[17]

Epithelial damage
0 Normal
1 Focal pathology
2 Extensive pathology
Architectural changes
0 Normal
1 Moderately (< 50%) disturbed
2 Severely (> 50%) disturbed
Infiltration of mononuclear cells in the lamina propria
0 Normal
1 Moderate increase
2 Severe increase
Infiltration of polymorphonuclear cells in the lamina propria
0 Normal
1 Moderate increase
2 Severe increase
Infiltration of polymorphonuclear cells in the epithelium
0 Normal
1 If in surface epithelium
2 If cryptitis
3 If a crypt abscess present
Presence of erosion or ulcers
0 No
1 Yes
The number of biopsy specimens affected
0 None
1 < 33%
2 33%-66%
3 > 66%

The score was calculated separately for each segment.

Ethical considerations

The families were informed about the study, and a written informed consent was obtained from the patient and their guardians. The study protocol was approved by the Ethics Committee for the Hospital for Children and Adolescents, University of Helsinki.

RESULTS

The CRP levels in paediatric IBD patients at the time of the colonoscopy

Standard CRP testing was undetectable (< 5 mg/L) in 64% (25/39) paediatric IBD patients investigated at the time of the colonoscopy. Of the patients with standard CRP levels below the detection limit, hs-CRP was measurable in all cases (Figure 1).

The median CRP level in the whole IBD group was 0.4 mg/L (0.007-45 mg/L). In all IBD patients, either with a fresh (0.7 mg/L, 0.02-39) or existing (0.2 mg/L, 0.007-45) diagnosis of colitis, CRP levels were higher than in the control patients ($P < 0.01$, see below). However, in patients with standard CRP levels under the detection limit of 5 mg/L, the hs-CRP measurement could

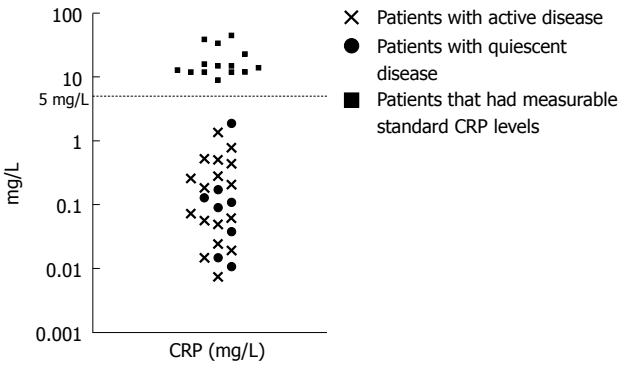


Figure 1 The C-reactive protein (CRP) levels in the 39 paediatric inflammatory bowel disease (IBD) patients undergoing colonoscopy. 14 patients had measurable (> 5 mg/L) standard CRP levels, the high-sensitivity CRP (hs-CRP) levels were analyzed in 25 patients that had undetectable standard CRP. For hs-CRP the detection limit is 3×10^{-6} mg/L.

not stratify the patients according to disease activity (active disease 0.2 mg/L, 0.007-1.37, $n = 17$ *vs* quiescent disease 0.1 mg/L, 0.01-1.89, $n = 8$, P = NS).

In CD patients, standard CRP was undetectable in 45% (9/20) of the patients, in UC in 84% (16/19) of the patients ($P < 0.05$). Patients with ileocolonic CD had clearly higher CRP levels (14 mg/L, 0.06-45, $n = 13$) than patients with CD colitis (0.18 mg/L, 0.01-9, $n = 7$, $P < 0.01$) whose CRP levels were similar to the patients with UC (0.13 mg/L, 0.007-23, P = NS). The CRP levels in CD patients were also higher in those presenting with granulomas (14 mg/L, 0.5-39, $n = 9$) than in those without granulomas (0.5 mg/L, 0.01-45, $n = 11$, $P < 0.05$). However, patients with granulomas did not have significantly more ileocolonic disease than colitis ($P = 0.07$).

The correlations between CRP levels, inflammatory markers and histology score, and the median (range) of these variables are presented in Table 4. The CRP levels did not correlate with age or weight; however, boys had higher CRP levels (4.9 mg/L, 0.007-45, $n = 24$) than girls (0.2 mg/L, 0.01-14, $n = 15$, $P < 0.05$).

The CRP levels in the non-IBD controls

The median CRP level in the 33 non-IBD controls was 0.03 mg/L (0.008-0.7 mg/L), significantly lower that in the above-mentioned group of 39 IBD patients ($P < 0.001$). In controls, the distribution of age (12.1 years, 1.8-17.2), sex (20 males, 13 females) and weight (37 kg, 12.8-84.6) were comparable to the 39 IBD patients (P = NS). Unlike in the IBD patients, CRP in the control patients correlated with weight ($r = 0.460$, $P < 0.01$). The control patients in the highest hs-CRP quartile were admitted due to obesity ($n = 1$), chronic autoimmune hepatitis ($n = 1$), leukopenia ($n = 1$), hypercholesterolemia ($n = 1$), talassemia minor ($n = 1$), gastro-oesophageal reflux disease ($n = 1$), coeliac disease ($n = 1$) and growth follow-up ($n = 1$).

The CRP levels in children with IBD during glucocorticoid therapy

The median pre-treatment CRP level of the 22 IBD patients starting peroral glucocorticoid therapy was 0.6 mg/L (0.01-39), similar to the level of CRP in the 39 IBD patients undergoing colonoscopy (P = NS). At the onset of the glucocorticoid treatment, the standard CRP levels were below the detection limit of 5 mg/L in 59% (13/22) of the patients. After 2-4 wk of treatment, the median level was 0.08 mg/L (0.004-60, $P < 0.05$). Of the 13 patients whose hs-CRP was measured (standard CRP undetectable), 7 patients responded well to the glucocorticoids; however the change in their hs-CRP levels during the glucocorticoid did not differ from those patients who had poor response ($P = 0.16$). The development of glucocorticoid-related side effects was not associated with serum CRP levels (data not shown).

Table 4 The median (range) levels of inflammatory markers and histology score and their correlation with the CRP levels in 39 paediatric patients with IBD

	Median (range)	Correlation with CRP
Inflammatory markers		
Calprotectin ($n = 35$)	690 μg/g (14-4400)	0.488[b]
ESR ($n = 37$)	18 mm/h (2-97)	0.618[d]
WBC ($n = 38$)	7.6 E9/L (3.2-17.4)	-0.011
Histology score		
Ileum ($n = 29$)[1]	0 (0-15)	0.475[a]
Caecum ($n = 38$)	4 (0-14)	-0.036
Ascendens ($n = 34$)	4.5 (0-13)	-0.198
Transversum ($n = 36$)	6 (0-15)	-0.158
Descendens ($n = 39$)	6 (0-15)	-0.077
Sigma ($n = 35$)	5 (0-14)	-0.014
Rectum ($n = 38$)	5.5 (0-14)	-0.051
Total colon ($n = 32$)	30 (0-74)	-0.083

[1]The histology score of the ileum was positive in only one UC patient (with backwash ileitis), all the other positive scores are from CD patients. [a]$P < 0.05$, [b]$P < 0.01$, [d]$P < 0.001$. ESR: Erythrocyte sedimentation rate; WBC: White blood cell count.

DISCUSSION

The aim of our study was to analyse whether hs-CRP can improve the assessment of disease activity in paediatric patients with IBD. We measured CRP with standard and high-sensitivity methods in children and adolescents presenting with colitis, and studied the association with other inflammatory markers (ESR, faecal calprotectin, and white blood cell count) and with the histological activity of the inflammation in the gut.

Even though CRP is commonly used as a marker of inflammatory activity in IBD patients, our findings support the concept that a significant number of patients with active IBD present with low CRP levels[6,10]. Here, standard CRP was under the detection limit of 5 mg/L in 60% of the paediatric patients with active colitis. All patients with undetectable standard CRP had measurable hs-CRP levels but there was no cut-off value for low CRP values with which to differentiate active and quiescent disease.

In this study all patients with CD presented with colitis. Thereby, it was somewhat surprising that there was a clear difference in the CRP values between UC and CD, the latter presenting with higher values. However, high CRP values associated with ileocolonic CD and

in CD colitis with no ileal involvement the CRP values were similar to UC. This is in contrast to previous studies in adults with IBD showing no difference between disease location and CRP levels in CD patients, or even opposing results - ileal disease associated with low CRP levels[6,20]. However, in our study we investigated also the association between CRP and histological activity of the colonoscopy biopsies, and found that the CRP levels correlated only with the histological score of the ileal biopsies, not with colonic inflammation. Studies in adults with CD have shown that an accumulation of intra-abdominal fat has been observed in CD patients, and that CRP levels correlate with radiological findings of small bowel perienteric inflammation (increased fat density)[21,22]. Since CRP is expressed in human adipose tissue, our findings may reflect higher local CRP production in the inflamed perienteric ileal region[23].

The association between granulomas and CRP levels in our study is a novel observation. In the literature, the evidence on the clinical significance of granulomas in CD patients is conflicting. Some studies associate the presence of granulomas with a more aggressive disease course, whereas others do not report any connection between granulomas and disease severity[24,25]. Recently, a study from the Children's Hospital of Philadelphia did not find a relationship between CRP levels and granulomas, although the presence of granulomas was associated with a more severe disease course[26]. The discrepancy between the CRP findings in these two studies could be explained by the fact that the patient populations differ, because the US study also included patients presenting granulomas only in the upper GI tract or terminal ileum. In an *in vitro* study, invasive *Escherichia coli* strains recovered from CD lesions caused macrophages to secrete TNF-α[27]. It is intriguing to speculate that higher local production of TNF-α in the granulomatous lesions of CD patients could explain the higher CRP levels associating with granulomas in our study. However, as yet the relationship remains elusive.

Only a few groups have studied the hs-CRP levels in IBD, and none of them have concentrated on the treatment-induced changes[11,28,29]. In this study, we additionally analysed a group of children and adolescents with IBD that started peroral glucocorticoid therapy due to the activation of the disease. In our small group of patients however, the measurement of hs-CRP did not provide any additional information as to the treatment of these patients.

Limitations of our study are the relatively small sample size, especially when the patients are divided into subgroups of UC and CD. In addition, apart from inflammation, minor trauma, acute infection, obesity and the use of NSAIDs, and genetic heterogeneity in the basal expression of CRP may affect the hs-CRP levels[30]. However, all patients undergoing colonoscopy had normal BMI and did not have any clinical signs of acute infection. As the subjects are children, we cannot rule out the possibility of minor bumps and bruises that might have affected the hs-CRP levels. Also, the possible intake of NSAIDs was unfortunately not recorded.

In conclusion, our study reinforces the concept that a significant number of paediatric patients with active IBD may present with CRP levels that are under the detection limit. Hs-CRP instead, was detectable in all the patients. Unfortunately, in this pilot study the measurement of hs-CRP levels in the patients that had undetectable standard CRP levels could not stratify the patients according to disease activity or response to treatment.

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COMMENTS

Background

C-reactive protein (CRP) is used to assess disease activity in diverse inflammatory disorders including inflammatory bowel disease (IBD). However, in IBD a significant number of patients present with low CRP levels despite clinically active disease. In paediatric patients with IBD the performance of CRP is an understudied area.

Research frontiers

High-sensitivity CRP (hs-CRP) measures CRP levels that were previously thought to be under the detection limit. In paediatric IBD, this kind of highly sensitive marker is needed for the detection of the presence of inflammation. Thus, the authors studied the performance of hs-CRP in paediatric IBD to see whether it improves the assessment of inflammation in these patients and/or associates with the response to glucocorticoid therapy.

Innovations and breakthroughs

Hs-CRP is a sensitive marker of inflammation that is used in various conditions (asthma, cardiovascular disease). In paediatric IBD, the performance of the hs-CRP has not been studied previously. The authors show here that standard CRP test is negative in a considerable number of paediatric patients with active IBD and the routine measurement of CRP is thus not informative enough. Hs-CRP detects low levels of CRP but disappointingly it does not help to distinguish children with active intestinal inflammation from those with quiescent disease or those responding to glucocorticoid treatment from non-responders. Interestingly, the levels of hs-CRP correlated with the presence of ileal inflammation.

Applications

The determination of hs-CRP seems to be of minor aid in assessing the presence of inflammation in paediatric IBD. In ileal disease, however, hs-CRP correlates with the presence of histological inflammation, a finding warranting further studies.

Terminology

IBD comprising Crohn's disease (CD) and ulcerative colitis is a chronic, relapsing and remitting disease of the gastrointestinal tract characterized by diarrhea, abdominal pain and weight loss. CRP is an acute phase protein produced by liver that is used in the diagnosis of inflammatory disorders.

Peer review

This clinical study has originality, is well designed and executed; the results are well presented and interpreted. Although this study shows a negative result, it has still provided valuable information on the CRP measurement in children IBD patients. One of the weaknesses is the small sample size of subgroup patients particularly CD patients in Table 2. It would strengthen the conclusion if more patients were included in this study.

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BRIEF ARTICLE

Endoscopic diagnosis of cytomegalovirus gastritis after allogeneic hematopoietic stem cell transplantation

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Abstract

AIM: To clarify the endoscopic and clinical findings of cytomegalovirus (CMV) gastritis after allogeneic hematopoietic stem cell transplantation (allo-SCT).

METHODS: Between 1999 and 2005, 523 patients underwent allo-SCT at our hospital, and 115 of these patients with gastrointestinal symptoms underwent esophagogastroduodenoscopy.

RESULTS: CMV gastritis was diagnosed pathologically in seven patients (1.3%) with the other 108 patients serving as controls. Six of the seven patients developed positive CMV antigenemia, and five complained of abdominal pain. Development of abdominal pain preceded CMV antigenemia in four of the five patients. Endoscopic examination showed oozing (n = 2), erosion (n = 6), and redness (n = 5) in the seven patients with CMV gastritis, while the control patients showed oozing (n = 3), erosion (n = 24), and redness (n = 100). Erosion and oozing were more frequently documented in patients with CMV gastritis compared with the controls, and the differences were statistically significant (P = 0.0012 and 0.029, respectively). CMV inclusion bodies were documented in 12 of 14 biopsy specimens obtained from erosive lesions, while they were identified in 4 of 15 biopsy specimens obtained from lesions other than erosions (P = 0.0025).

CONCLUSION: This study suggests that erosion and oozing, as well as abdominal pain, are useful indicators in the diagnosis of CMV gastritis following allo-SCT.

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Key words: Cytomegalovirus gastritis; Hematopoietic stem cell transplantation; Cytomegalovirus antigenemia; Esophagogastroduodenoscopy; Graft-versus-host disease

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INTRODUCTION

Cytomegalovirus (CMV) disease is a serious complication after allogeneic hematopoietic stem cell transplantation (allo-SCT)[1], which is widely accepted as a curative therapy for advanced hematological malignancies including leukemia and malignant lymphoma. CMV disease can involve many organs and the gastrointestinal (GI) tract is a common target[2].

CMV antigenemia is one of the most widely used methods to detect CMV reactivation in a variety of clinical settings[3]; however, it is of limited value in predicting and diagnosing GI CMV disease[4]. GI CMV disease is usually diagnosed based on pathological examination of endoscopically obtained mucosal biopsy specimens. Few reports have been published regarding endoscopic examination in diagnosing CMV gastritis after allo-SCT[5-7]. This study aimed to investigate endoscopic findings of CMV gastritis after allo-SCT in addition to its clinical features.

MATERIALS AND METHODS

Study patients

Between January 1999 and September 2005, 523 patients underwent allo-SCT at the National Cancer Center Hospital in Tokyo, Japan. Among them, 115 patients with GI symptoms underwent esophagogastroduodenoscopy (EGD). Written informed consent was obtained from all patients before EGD. We retrospectively reviewed records of medical, endoscopic and pathological examination in the 115 EGD patients. CMV gastritis was diagnosed pathologically in seven patients (1.3%) by hematoxylin-eosin staining and immunohistochemical staining with an anti-CMV antibody. The other 108 patients served as controls.

Endoscopic procedure

All EGD patients orally received 100 mL of a solution containing 1 g of pronase and 1 g of sodium bicarbonate to remove mucus and bubbles on the gastric mucosa before EGD. Antiperistaltic agents (scopolamine butylbromide 20 mg or glucagon 1 mg) and sedatives (pethidine hydrochloride 17.5-35 mg or midazolam 2-3 mg) were injected intravenously. Conventional endoscopic instruments (GIF Q240; Olympus Co, Ltd, Tokyo, Japan) were used, and biopsy specimens were obtained endoscopically from severely involved areas. When abnormal findings were not found, biopsy specimens were obtained from normal appearing areas.

Pathological examination

Biopsy specimens were fixed immediately in a 10% buffered formalin solution and subsequently stained with hematoxylin-eosin. All tissues were examined by expert pathologists. Diagnosis of CMV gastritis was based on histological identification of CMV inclusion bodies by hematoxylin-eosin staining and immunohistochemical staining with an anti-CMV antibody. Diagnosis of graft-versus-host disease (GVHD) was determined in accordance with a report published previously[8].

Management of CMV

All patients were monitored at least once a week for CMV reactivation by CMV antigenemia assay using monoclonal antibody against C7-HRP (Teijin, Tokyo, Japan) after engraftment.

A patient was considered to be infected with CMV when CMV antigenemia assay detected CMV in the blood. A patient was considered to have CMV disease when CMV was demonstrated in biopsy specimens by hematoxylin-eosin staining and immunohistochemical analysis. Ganciclovir was initiated when either more than 10 cells per 50 000 cells were positive according to the CMV antigenemia assay in patients transplanted from related donors, a single cell per 50 000 cells was positive in patients transplanted from unrelated donors, or a patient was diagnosed as having CMV disease[9].

Management of GVHD

Acute GVHD was graded according to the consensus criteria[10,11] and all patients with grades Ⅱ-Ⅳ acute GVHD were treated with 0.5-2.0 mg/kg per day of methylprednisolone.

Statistical analysis

Univariate analysis using Fisher's exact test was performed to compare differences in patient characteristics, clinical features, and endoscopic findings between the seven patients with CMV gastritis and the other 108 patients who had GI symptoms, but did not have CMV gastritis. Values of $P < 0.05$ were considered significant.

RESULTS

Patient characteristics

Patient characteristics are shown in Table 1. There was a significant difference in the number of patients given tacrolimus with methotrexate as GVHD prophylaxis between the two groups ($P = 0.018$).

Clinical features

Five of the seven patients with CMV gastritis complained of abdominal pain, while 31 of the 108 control patients complained of abdominal pain ($P = 0.030$) (Table 2). The pain was localized in the upper abdomen in all four patients with CMV gastritis whose medical reports provided the specific location of their pain (Table 3). Three patients required significant analgesia (morphine hydrochloride for one and pentazocine hydrochloride for the other two). Abdominal pain improved with ganciclovir in four of the five patients with abdominal pain, and the remaining patient (Case 1) died of bacterial pneumonia without any improvement in CMV gastritis.

Watery diarrhea was found in four of the seven patients with CMV gastritis, and was complicated by intes-

Table 1 Patient characteristics with and without CMV gastritis

Variables		Patients with CMV gastritis (n = 7)	Patients without CMV gastritis (n = 108)
Median age (range)		47 (26-62)	45 (18-69)
Gender	Male/female	5/2	65/43
Underlying diseases	Acute leukemia	1	41
	Chronic leukemia	2	15
	Malignant lymphoma	3	21
	Myelodysplastic syndrome	1	22
	Others	0	9
Preparative regimens	Myeloablative/reduced-intensity	2/5	48/60
Stem cell sources	Marrow/peripheral blood/cord blood	3/3/1	39/64/5
GVHD prophylaxis	CSP alone/CSP + MTX/CSP + MMF/ FK506 + MTX/FK506 Alone	2/3/0/2[a]/0	36/64/2/2[a]/4

CMV: Cytomegalovirus; GVHD: Graft-versus host disease; CSP: Cyclosporine; MTX: Methotrexate; MMF: Mycophenolate mofetil; FK506: Tacrolimus. [a]P = 0.018.

Table 2 Clinical features in patients with and without CMV gastritis

Variables			Patients with CMV gastritis (n = 7)	Patients without CMV gastritis (n = 108)
Gastrointestinal symptoms at EGD		Nausea	2	60
		Vomiting	1	26
		Abdominal pain	5[a]	31[a]
		Abdominal discomfort	2	23
		Hematemesis	1	2
		Tarry stool	2	6
		Watery diarrhea	4	32
		Appetite loss	0	40
CMV	Median onset of CMV gastritis	Days (range)	63 (33-167)	NA
	CMV antigenemia (C7-HRP) at EGD	Positive/negative/not done	6[b]/1/0	28[b]/71/9
		Median number of positive cells per 50000 (range)	8[d] (0-143)	0[d] (0-167)
	Involved organs of CMV diseases	Esophagitis/duodenitis/enterocolitis/ pneumonitis/retinitis	1/2/1/0/0	0/0/4/0/0
GVHD		Positive (clinical grade: Ⅰ/Ⅱ/Ⅲ/Ⅳ)	7[c] (2/2/3/0)	65[c] (45/10/9/1)

EGD: Esophagogastroduodenoscopy; NA: Not applicable. [a]P = 0.030, [b]P = 0.0026, [c]P = 0.044, [d]P = 0.0023.

Table 3 Clinical features of CMV gastritis

	Demographics	Gastrointestinal symptoms			CMV antigenemia assay	
	Age (yr), gender, diagnosis	Any symptoms	Abdominal pain		Onset (d)	Level at EGD (cells per 50000)
			Onset (d)	Localization in abdomen		
Case 1	34, male, CML	Abdominal pain, tarry stool	81	Upper abdomen	88	8
Case 2	43, female, MDS	Nausea, abdominal pain, tarry stool, hematemesis, watery diarrhea	53	No description	69	2
Case 3	60, male, AML	Abdominal pain, watery diarrhea	62	Upper abdomen	73	143
Case 4	48, female, ML	Abdominal pain, watery diarrhea	36	Upper abdomen	31	10
Case 5	47, male, ML	Abdominal pain, watery diarrhea	30	Upper abdomen	32	4
Case 6	62, male, CML	Abdominal discomfort	NA	NA	47	32
Case 7	26, male, ML	Nausea, vomiting, abdominal discomfort	NA	NA	NA	0[1]

[1]CMV antigenemia remained negative throughout clinical course. CML: Chronic myelocytic leukemia; MDS: Myelodysplastic syndrome; AML: Acute myelocytic leukemia; ML: Malignant lymphoma.

tinal GVHD in three of these four patients. Watery diarrhea improved with ganciclovir in a patient with CMV gastritis who had no evidence of intestinal GVHD.

All seven patients with CMV gastritis had GVHD, while 65 of the 108 control patients had GVHD (P = 0.044) (Table 2). Five of the seven patients with CMV gastritis had grade Ⅱ-Ⅳ GVHD that was being treated by corticosteroids.

CMV antigenemia assay

Six of the seven patients with CMV gastritis and 28 of the 108 controls showed positive CMV antigenemia (P =

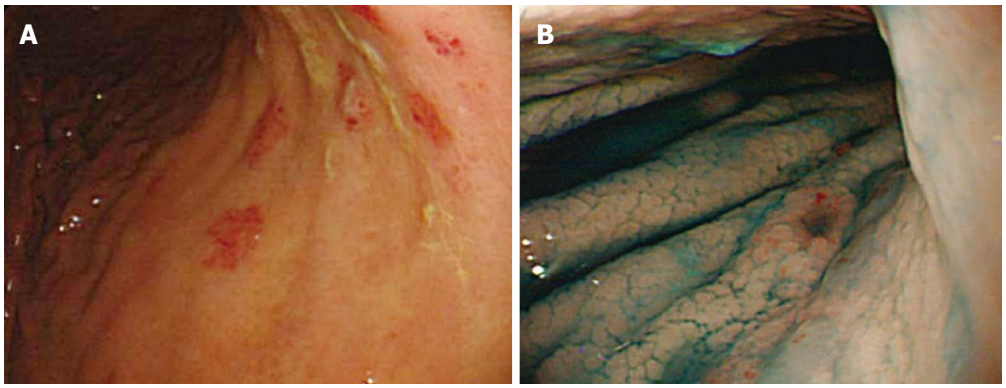


Figure 1 Erosion (Case 6). Multiple erosions are clearly shown in gastric body. A: Before indigo carmine dye spraying; B: After indigo carmine dye spraying).

Table 4 Endoscopic features in patients with and without CMV gastritis

Variables	Patients with CMV gastritis (n = 7)	Patients without CMV gastritis (n = 108)
Atrophic mucosa	3	36
Redness	5	100
Edema	2	9
Orange peel appearance	2	21
Mucosal sloughing	1	6
Erosion	6[b]	24[b]
Ulceration	0	2
Oozing	2[a]	3[a]

[a]P = 0.029, [b]P = 0.0012.

0.0026) (Table 2). The median number of positive cells in the CMV antigenemia test among the seven patients with CMV gastritis was 8 cells per 50 000 cells (range, 0-143) at the time of EGD.

Development of abdominal pain preceded the CMV antigenemia in four of the five patients who complained of it, and the median interval between onset of abdominal pain and the first positive CMV antigenemia was 7 d (range, -5 to 16 d) (Table 3).

Endoscopic findings

Erosion was observed in six of the seven patients with CMV gastritis and in 24 of the 108 control patients (P = 0.0012) (Table 4). The erosive lesions were located in the antrum (n = 2), body (n = 2), and antrum-body (n = 2) of the stomach. Two of the six patients had a solitary erosion, and the other four patients had multiple erosions of various sizes. Erosions were flat in four patients and raised in the other two. A representative example of erosion is shown in the accompanying figure; multiple erosions are clearly shown in the gastric body (Figure 1).

Oozing was observed in two of the seven patients with CMV gastritis and in three of the 108 control patients (P = 0.029). Oozing was located in the antrum with erosion (Case 3), and in the antrum-body with mucosal sloughing (Case 2).

Pathological findings

Detailed information regarding pathological findings is shown in Table 5. CMV inclusion bodies were documented in 12 of 14 biopsy specimens obtained from erosive lesions, while they were identified in 4 of 15 biopsy specimens obtained from lesions other than erosions (P = 0.0025) (Table 5).

Outcomes

Four patients died, and CMV disease was not the primary cause of death in any of them (Table 5). Two died from recurrences of their primary diseases, one died of bacterial pneumonia and one died of renal failure.

DISCUSSION

The present study clarifies the endoscopic findings of CMV gastritis following allo-SCT in addition to its clinical features. CMV gastritis was diagnosed pathologically in seven patients (1.3%) among 523 patients who underwent allo-SCT at our facility. The incidence is comparable to a previous study (1.7%)[12]. None of the seven patients died of CMV gastritis, while three patients complained of significant abdominal pain requiring analgesia which impaired their quality of life. CMV gastritis was a clinically important complication after allo-SCT.

No detailed information on clinical features of CMV gastritis has been previously reported. In the present study, abdominal pain was a common symptom of CMV gastritis. The pain was localized in the upper abdomen in all four patients whose medical reports provided the specific location of their pain. Ganciclovir administration improved abdominal pain in these four patients, supporting the likelihood that this symptom was attributable to CMV gastritis. Clinicians should pay particular attention to upper abdominal pain following allo-SCT as a possible symptom of CMV gastritis.

The association between watery diarrhea and CMV gastritis may be minimal as it remained unclear whether such diarrhea was due to CMV gastritis or overlapping intestinal GVHD. In the present study, ganciclovir improved symptoms in only one of four patients with diarrhea. In contrast, CMV gastritis was complicated by intestinal GVHD in three of those four patients. Our observations suggested that watery diarrhea in patients with CMV gastritis was more likely due to intestinal GVHD rather than the CMV gastritis itself.

Endoscopic findings characteristic of CMV gastritis

Table 5 CMV inclusion bodies and response to ganciclovir of CMV gastritis

	Positive specimens with CMV inclusion bodies/total specimens in EGD biopsy				Response to ganciclovir		Outcome	
	Total	Erosions	Mucosal sloughing	Other findings or normal mucosa	Abdominal pain	CMV antigenemia assay	Outcome	Cause of death
Case 1	1/2	1/2	None	0	Continued	Continued	Death	Bacterial pneumonia
Case 2	2/2	None	2/2[1]	0	Improved	Turned negative	Death	Recurrence of primary disease
Case 3	2/3	2/3[1]	None	0	Improved	Turned negative	Death	Renal failure
Case 4	5/7	4/4	None	1[2]/3	Improved	Turned negative	Alive	NA
Case 5	1/5	1/1	None	0/4	Improved	Turned negative	Alive	NA
Case 6	4/7	3/3	None	1[2]/4	NA	Turned negative	Alive	NA
Case 7	1/3	1/1	None	0/2	NA[3]	NA[4]	Death	Recurrence of primary disease
Case 7		26, male, ML		Nausea, vomiting, abdominal discomfort	NA	NA	NA	0[1]

[1]Oozing was accompanied in these findings; [2]The patient was not given ganciclovir, but CMV gastritis improved spontaneously; [3]The patient was not given ganciclovir, and CMV antigenemia remained negative throughout clinical course; [4]The patient was not given ganciclovir, and CMV antigenemia remained negative throughout clinical course.

after allo-SCT have not been fully investigated, but the present study indicates that erosion and oozing might be useful markers for early diagnosis of CMV gastritis. Vascular endothelium infected with CMV narrows vessels and induces local ischemia[13] eventually resulting in erosions and oozing. In fact, most CMV inclusion bodies were obtained from erosion sites. Erosions from CMV gastritis developed in all stomach sites and varied in size. Endoscopists should suspect CMV gastritis and obtain multiple biopsies whenever erosions are found in any stomach site.

In contrast, none of the seven patients with CMV gastritis had punched out ulcers which had previously been considered characteristic of GI CMV disease[14-16]. In the present study, early EGD might have enabled early diagnosis of CMV gastritis before progression to ulcers. In two patients (Cases 4 and 6), CMV inclusion bodies were identified pathologically from normal mucosa as well as erosions. This result demonstrates the necessity of biopsy even if only normal findings are identified when EGD is performed.

CMV antigenemia reflects the severity of CMV reactivation[3,17], but the clinical significance of CMV antigenemia remains unknown in the diagnosis of GI CMV disease because of the wide variation in positive findings, ranging from a low of 21%[4] to a high of 73%[18]. In this study, CMV antigenemia was positive in six of the seven patients with CMV gastritis. This result supports the usefulness of CMV antigenemia in the diagnosis of CMV gastritis. It should be noted that abdominal pain preceded CMV antigenemia in four of the five patients with positive CMV antigenemia and abdominal pain. Our observations suggest that elaboration of physical and endoscopic examinations is even more important than detection of CMV antigenemia in the early diagnosis of CMV gastritis.

Patients with GVHD, and patients given corticosteroids for treatment of GVHD, carry a high risk of CMV disease[19]. In this study, such increased risk was confirmed as all seven patients with CMV gastritis also had GVHD and five of them had grade Ⅱ-Ⅳ GVHD that was being treated by corticosteroids. GVHD, by itself and also accompanied by corticosteroid administration, are exacerbating factors in the existence of CMV gastritis.

The present investigation was a retrospective study based on our examination of medical records as well as endoscopic and pathological findings. The small size of the study does not exclude the possibility of unrecognized bias. Since EGD was not conducted in all allo-SCT recipients, underestimation of the frequency of CMV gastritis is a possibility. Consequently, further prospective evaluation is warranted to clarify the endoscopic findings for early diagnosis of CMV gastritis.

The results of this study suggest that endoscopic and clinical findings are useful indicators in the diagnosis of CMV gastritis following allo-SCT. Use of EGD is warranted for the establishment of an early diagnosis of CMV gastritis following allo-SCT.

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COMMENTS

Background

Cytomegalovirus (CMV) disease is a serious complication after allogeneic hematopoietic stem cell transplantation (allo-SCT), which is widely accepted as a curative therapy for advanced hematological malignancies including leukemia and malignant lymphoma. CMV disease can involve many organs and stomach is a common target.

Research frontiers

Few reports have been published regarding endoscopic examination in diagnosing CMV gastritis after allo-SCT. In this study, the authors demonstrate the endoscopic findings of CMV gastritis after allo-SCT in addition to its clinical features.

Innovations and breakthroughs

The present study indicated that erosion and oozing might be useful markers for early diagnosis of CMV gastritis.

Applications

Endoscopists should suspect CMV gastritis and obtain multiple biopsies whenever erosions are found in any stomach site when performing esophagogastroduodenoscopy in patients after allo-SCT.

Peer review

This is an interesting manuscript on an important topic. The study presented here is a retrospective one with a small number of affected patients, but it offers some deeper insights into this complex problem. Limitations of this study and difficulties in diagnosis and interpretation are addressed by the authors in the discussion section.

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BRIEF ARTICLE

Risk factors for bleeding after endoscopic submucosal dissection for gastric lesions

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Abstract

AIM: To assess risk factors for bleeding after gastric endoscopic submucosal dissection (ESD) and to develop preventive measures.

METHODS: This retrospective study was performed in a tertiary referral center. A total of 328 patients underwent ESD for 398 gastric neoplasms between July 2007 and June 2009. The main outcome was association between post-ESD bleeding and the following: age; sex; comorbidities; daily use of medicine potentially related to gastric injury/bleeding; location, size, and histological depth of lesions; ulceration; experience of operator coagulating the ulcer floor, and duration of operation. We also determined the relationship between the location of post-ESD bleeding and risk factors for hemorrhage.

RESULTS: Univariate analysis revealed significant risk factors: tumor location [odds ratio (OR), 2.86; 95% CI: 1.21-6.79, P = 0.024], coagulator experience (OR, 4.29; 95% CI: 1.43-12.86, P = 0.009), and medicine potentially related to gastric injury/bleeding (OR, 2.80; 95% CI: 1.14-6.90, P = 0.039). Multivariate logistic regression analysis confirmed significant, independent risk factors: tumor in lower third of stomach (OR, 2.47; 95% CI: 1.02-5.96, P = 0.044), beginner coagulator (OR, 3.93; 95% CI: 1.29-11.9, P = 0.016), and medicine (OR, 2.76; 95% CI: 1.09-6.98, P = 0.032). We classified cases of post-ESD bleeding into two groups (bleeding at the ulcer margin *vs* bleeding at the center) and found that bleeding at the margin occurred more frequently with beginner coagulators compared with experts (OR, 16.00; 95% CI: 1.22-210.59, P = 0.040).

CONCLUSION: Beginner coagulators, tumor in the antrum, and medicines were significant risk factors for post-ESD bleeding. Bleeding at the ulcer margin frequently occurred with beginner operators.

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Key words: Bleeding; Endoscopic submucosal dissection; Ulcer floor; Anti-thrombotic drugs; Coagulation

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INTRODUCTION

Endoscopic submucosal dissection (ESD) is an excellent treatment for superficial gastric cancer because a large tumor or lesion with ulcer scar can be successfully resected *en bloc*[1]. Because ESD allows accurate histopathological diagnosis and reduces the risk of local recurrence[2], it is now a standard treatment for early gastric cancer with certain histopathological properties. However, ESD is technically more difficult and can result in more complications compared with conventional endoscopic mucosal resection (EMR)[1,3]. Bleeding after gastric ESD is reported to occur in up to 7% of cases[4] and can occur later than other complications like perforation, sometimes even after discharge from the hospital. Further, bleeding after gastric ESD may lead to serious conditions including massive bleeding and life-threatening hemorrhagic shock[5]; therefore, preventing post-gastric ESD bleeding is essential. Prophylactic coagulation of visible vessels in the resection area has been reported to reduce the bleeding rate[5], but preventive coagulation therapy cannot fully solve the problem of delayed bleeding. Thus, an assessment of risk factors for post-ESD bleeding is needed.

In the present study, we aimed to determine risk factors for post-gastric ESD bleeding and establish techniques to minimize these complications.

MATERIALS AND METHODS

Patients

A total of 329 patients underwent ESD for 399 gastric neoplasms, including early gastric cancers and gastric adenomas, between July 2007 and June 2009 in our institute. One lesion was excluded from the analysis because ESD for that lesion was stopped halfway during the procedure; it was considered unresectable by ESD because of massive submucosal invasion. Thus, 398 lesions in 328 patients were evaluated.

ESD procedure

All patients provided written informed consent before treatment. Patients fasted on the morning of the operation, and lansoprazole infusion (30 mg twice a day) was initiated. ESD was performed under conscious sedation with flunitrazepam and buprenorphine using a video endoscope (GIF-Q260J; Olympus Optical Co., Ltd., Tokyo, Japan) and high-frequency power supply unit (VIO 300D or ICC 200; ERBE, Tübingen, Germany).

The ESD procedure was as follows. First, marks were made 5 mm outside the tumor edge with a Flex Knife (Olympus Optical Co., Ltd.). Epinephrine (1:100 000 solution in saline) was injected into the submucosal layer around the lesion, and the mucosa 5 mm outside the marks was cut using IT-Knife 2 (Olympus Optical Co., Ltd.). After incision of the mucosa, submucosal dissection of the lesion was performed using IT-Knife 2. After resection of the lesion, all visible vessels on the ulcer floor were coagulated with hot biopsy forceps (Hoya Co., Ltd., Pentax Life Care Div., Japan) and VIO 300D (swift coagulation, effect 3, 45 W) or ICC 200 (forced coagulation, 65 W).

Patients who did not develop complications started a soft diet on day 1 after ESD and were discharged 5 d after the procedure. They received oral lansoprazole for 8 wk, beginning on day 1 after ESD. We defined bleeding after ESD as hemorrhage resulting in hematemesis or melena that required endoscopic treatment.

When hemorrhage occurred, we performed urgent endoscopy to determine the vessel responsible for the bleeding. When clots were lodged in the responsible vessel, they were removed and the responsible vessel was exposed. The vessel was grasped or touched with the hot biopsy forceps using the forced coagulation mode (ICC 200, 65 W).

Data analysis

We classified the 398 lesions into two groups: with bleeding and without bleeding. We reviewed patient medical records and collected the following data for each patient: age, sex, comorbidities, daily use of medicine related to gastric injury/bleeding, tumor depth, histological ulceration, tumor diameter, tumor location, and the operator performing coagulation of the ulcer floor (coagulator). The risk factors were analyzed based on individual tumors, because some patients had more than one lesion resected.

Comorbidities included hypertension (systolic blood pressure > 140 mmHg or antihypertensive treatment) and diabetes mellitus (oral hypoglycemic agent or insulin treatment). Medicines potentially related to gastric injury/bleeding included antiplatelet agents, anticoagulants, steroids, or nonsteroidal anti-inflammatory drugs (NSAIDs). Patients taking steroids or NSAIDs were instructed to discontinue their use 1 wk before ESD to 1 wk after ESD. Patients taking antiplatelet or anticoagulants were instructed to discontinue use according to "measures against patients undergoing antithrombotic regimen in endoscopic treatment" in the 3rd edition of the guidelines for gastroenterological endoscopy[6]. The cessation and restart of antithrombotic drugs in ESD is shown in Figure 1. Ulceration and depth were determined histopathologically after ESD; histological depth was classified as intramucosal (m) or involving the submucosal layer (sm). Based on the Japanese classification of gastric carcinoma[7], tumor location was classified into two groups: upper or middle third of the stomach (UM) or lower third (L). Coagulators were the physicians who coagulated the artificial ulcer floor after resection of the lesion. In our hospital, novice ESD operators begin their training with coagulation of the ulcer floor; therefore, the operator who resected the tumor was not always the one who coagulated the ulcer floor. Ulcer floor coagulators were categorized as beginners (performed < 50 cases of gastric ESD) or experts (performed > 200 cases of gastric ESD). In the present study, there were no coagulators who performed between 50 and 200 ESD procedures.

We also determined the relationship between the location of post-ESD bleeding and risk factors for hemorrhage. During the observation period, post-ESD bleeding occurred in 23 lesions (23 patients). The hemorrhage location

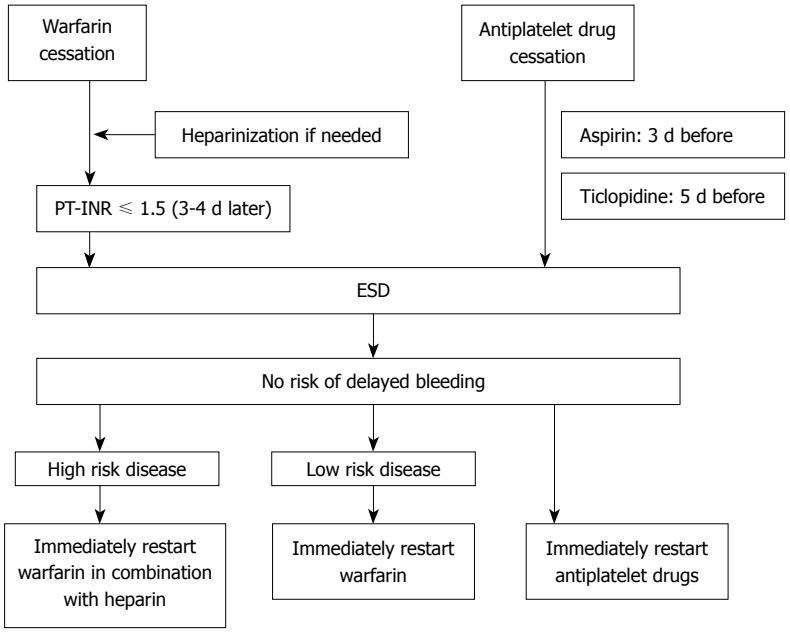


Figure 1 Flow chart summarizing the cessation and restart of antithrombotic drugs in endoscopic submucosal dissection (ESD)[6]. Warfarin is discontinued before ESD and heparin is initiated if needed. PT-INR is checked 3 to 4 d later; if PT-INR ≤ 1.5, ESD can be performed. After surgery warfarin is immediately restarted, and heparin is co-administered in patients at high risk for disease. Antiplatelet drugs are discontinued before ESD as well (aspirin, 3 d before ESD; ticlopidine, 5 d before ESD); they are immediately restarted after the procedure. PT-INR: Prothrombin time-international normalized ratio.

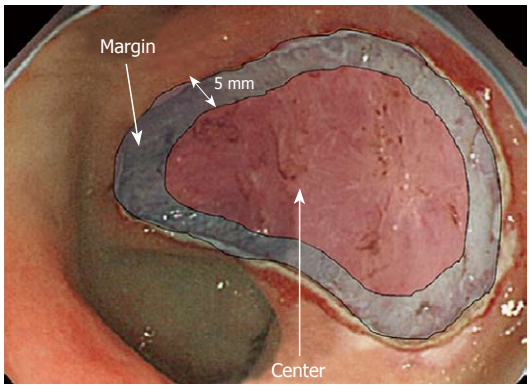


Figure 2 The margin and center areas of the ulcer floor. The margin includes the outer 5 mm edge of the ulcer, and the remaining area is defined as the center.

Table 1 Baseline characteristics of study population (n = 398)

	n (%)
Sex (M/F) (% M)	302/96 (75.6)
Age (yr)	68.0 ± 10.2
Hypertension (-/+) (% positive)	208/190 (47.7)
Diabetes mellitus (-/+) (% positive)	349/49 (12.3)
Drugs potentially related to gastric injury/bleeding (-/+) (% positive)	330/68 (17.1)
Location (UM/L) (% L)	252/146 (36.7)
Depth (m/sm) (% sm)	343/55 (13.8)
Ulcer (-/+) (% positive)	367/31 (7.8)
Diameter of lesion (cm)	43.1 ± 17.2
Duration of operation (min)	69.0 ± 56.7
Coagulator (expert/beginner) (% beginner)	182/216 (54.3)

Quantitative data are presented as mean ± SD.

was categorized as ulcer margin (< 5 mm from the ulcer edge) or center (ulcer floor area other than the margin) (Figure 2).

Statistical analysis

Categorical data were compared using χ^2 test (with Yates correction) and Fisher's exact test. Differences in the means of continuous data were compared by Student's *t* test or Mann-Whitney *U* test. To identify important risk factors for bleeding after ESD, predictors with *P*-values < 0.2 in the univariate analysis were included in a backward, stepwise multiple logistic regression model. *P*-value < 0.05 was considered significant, and all tests were two-sided. Data are expressed as mean ± SD or as median (range). All statistical analyses were performed with PASW Statistics 18 for Windows (SPSS Japan, Tokyo).

RESULTS

During the treatment of the 398 lesions, 23 cases of post-ESD bleeding occurred (5.8%). Baseline characteristics of the study population are displayed in Table 1. A total of six operators (two experts and four beginners) were involved in this study. Among the 23 cases of post-ESD bleeding, 10 (43.5%) occurred within 24 h after ESD. All cases were controlled by endoscopic treatment and did not require surgical intervention; one case required blood transfusion.

The results of univariate analysis are displayed in Table 2. Significant risk factors for bleeding after gastric ESD included: tumor location [L *vs* UM; odds ratio (OR), 2.86; 95% CI: 1.21-6.79, *P* = 0.024], coagulators (beginner *vs* expert; OR, 4.29; 95% CI: 1.43-12.86, *P* = 0.009), and daily use of medicine potentially related to gastric injury/bleeding (user *vs* non-user; OR, 2.80; 95% CI: 1.14-6.90, *P* = 0.039). As an additional datum, the incidence of hemorrhage in relation to tumor location was estimated (U *vs* M *vs* L; 4.6% *vs* 3.2% *vs* 9.6%, *P* = 0.042).

Multivariate logistic regression analysis confirmed that the following were independent factors for bleeding after gastric ESD: tumors located in the lower third of the stomach (OR, 2.47; 95% CI: 1.02-5.96, *P* = 0.044), begin-

Table 2 Background characteristics in relation to bleeding status (mean ± SD) *n* (%)

	Bleeding (-) (*n* = 375)	Bleeding (+) (*n* = 23)
Sex (M/F) (% M)	283/92 (75.4)	19/4 (82.6)
Age (yr)	67.8 ± 10.2	70.1 ± 10.0
Hypertension (-/+) (% positive)	198/177 (47.2)	10/13 (56.5)
Diabetes Mellitus (-/+) (% positive)	329/46 (12.2)	20/3 (13.0)
Drugs potentially related to gastric injury/bleeding (-/+) (% positive)	315/60 (16.0)	15/8 (34.8)[a]
Location (UM/L) (% L)	243/132 (33.2)	9/14 (60.9)[a]
Depth (m/sm) (% sm)	324/51 (13.6)	19/4 (17.4)
Ulcer (-/+) (% positive)	347/28 (7.4)	20/3 (13.0)
Diameter of lesion (cm)	42.7 ± 17.2	48.3 ± 17.3
Duration of operation (min)	68.8 ± 56.7	72.2 ± 56.4
Coagulator (beginner/expert) (% beginner)	197/178 (52.5)	19/4 (82.6)[a]

[a]$P < 0.05$ *vs* bleeding (-). *P* values by χ^2 test and Student's *t*-test.

Table 3 Significant predictors of post-ESD bleeding identified by multiple logistic regression

Characteristics	Odds ratio	95% CI	*P*
Drugs potentially related to gastric injury/bleeding	2.76	1.09-6.98[a]	0.032
Tumor in lower third of stomach	2.47	1.02-5.96[a]	0.044
Beginner coagulator	3.93	1.29-11.91[a]	0.016

[a]$P < 0.05$. ESD: Endoscopic submucosal dissection.

Table 4 Risk factors for post-ESD bleeding according to bleeding site

	Marginal bleeding (*n* = 17)	Central bleeding (*n* = 6)
Drugs potentially related to gastric injury/bleeding	6 (35.3)	3 (50.0)
Tumor in the lower third of stomach	9 (52.9)	5 (83.3)
Beginner coagulator	16 (94.1)	3 (50.0)[a]

[a]$P < 0.05$ *vs* marginal bleeding. *P* values by χ^2 test.

ner coagulators (OR, 3.93; 95% CI: 1.29-11.91, $P = 0.016$), and daily use of medicine potentially related to gastric injury/bleeding (OR, 2.76; 95% CI: 1.09-6.98, $P = 0.032$) (Table 3).

We classified cases of post-ESD bleeding into two groups (bleeding at the ulcer margin *vs* bleeding at the center), and determined the relationships between bleeding site and the three risk factors described above. We found that bleeding at the margin occurred more frequently with beginner coagulators compared with experts (OR, 16.00; 95% CI: 1.22-210.59, $P = 0.040$) (Table 4).

The interval between ESD and post-ESD bleeding was also determined. The median interval was 3 d (range, 0-14 d), and bleeding occurred significantly earlier in L lesions compared with UM lesions (UM *vs* L; 9 d (range, 0-14 d) *vs* 1 (0-9), $P = 0.019$). The relationship between patient medication and the interval between ESD and bleeding was also estimated. In the "medication" group, the median interval to bleeding was 3 d (range, 0-14 d), while in the "no medication" group, the median interval to bleeding was 1 d (range, 0-12 d). Based on the data medication did not appear to affect the interval between ESD and post-ESD bleeding ($P = 0.201$).

DISCUSSION

Previous studies[5,8-11] have evaluated measures against post-ESD bleeding. Takizawa *et al*[5] found that coagulating exposed vessels on the ulcer floor after ESD, which is known as post-ESD coagulation preventive therapy (PEC), reduced the risk for delayed bleeding. However, the incidence of bleeding after ESD was still 3.1% after PEC; therefore, additional measures are needed to prevent delayed bleeding.

In the present study, multivariate logistic regression analysis showed that tumors located in the lower third of the stomach, beginner operators coagulating the ulcer floor, and daily use of medicine potentially related to gastric injury/bleeding were significantly associated with bleeding after ESD.

Takizawa *et al*[5] also reported that tumor location was an independent risk factor for delayed bleeding. In that study, delayed bleeding occurred more frequently in the upper third compared with the middle and lower thirds of the stomach. In the present study, we compared UM lesions with L lesions, because we thought that antral active peristalsis and bile reflux might lead to a high incidence of post-ESD bleeding in the lower stomach. On the other hand, submucosal artery diameters were smaller in L lesions compared with UM lesions in a dog model[12]. For that reason, bleeding during gastric EMR may occur more frequently in UM lesions. Similarly, during ESD procedures, intraoperative bleeding frequently occurs in UM lesions. Intraoperative bleeding, however, may lead to more careful endoscopic hemostasis, which ultimately protects against delayed bleeding. In another study, an increased risk of delayed bleeding has been reported when immediate bleeding occurred during endoscopic mucosal resection[13]. However, sites of delayed bleeding often differed from the sites of immediate bleeding, suggesting that well-coagulated arteries seldom bleed.

Previous studies have reported that daily use of medicine potentially related to gastric injury/bleeding is not a risk factor for post-ESD bleeding[5,13]. However, multivariate logistic regression analysis in our study showed that use of these medicines was an independent risk factor for post-ESD bleeding. On the other hand, daily use of these drugs did not appear to affect the interval between ESD and bleeding. The median interval was 3 d, which was before medication was restarted; therefore, direct effects of these drugs on post-ESD bleeding appear to be limited. The mechanism underlying the association between daily use of medicine potentially related to gastric injury/bleeding and post-ESD bleeding is not known.

Our study also showed that post-ESD bleeding occurred significantly more often when beginners performed coagulation of the ulcer floor after ESD. The incidence

of hemorrhage was 2.2% when performed by experts, but 8.8% with beginners (Table 2). Beginners caused bleeding in the ulcer margin significantly more often. Coagulation of exposed vessels on the ulcer floor after ESD reduces the risk of hemorrhage, but potentially hemorrhagic vessels are often hidden in a fat layer that remains on the ulcer floor, especially at the edge of the ulcer. Thus, careful coagulation of vessels at the ulcer margin is important in preventing delayed bleeding.

In L lesions, hemorrhage occurred significantly earlier compared with UM lesions. The median interval between ESD and postoperative hemorrhage in L lesions was 1 d (range, 0-9 d), suggesting that second-look endoscopy 1 d after ESD may be helpful in preventing post-ESD bleeding in L lesions.

Limitations of the present study include its single-center retrospective study design.

In conclusion, risk factors for bleeding after gastric ESD included beginner operators coagulating the ulcer floor after ESD, tumor location in the lower third of the stomach, and medicine potentially related to gastric injury/bleeding. Careful coagulation of the vessels in the ulcer floor after ESD, especially around the ulcer margin, may reduce the incidence of post-ESD hemorrhage. In addition, a second-look endoscopy 1 d after ESD may be helpful in preventing post-ESD bleeding in tumors in the lower third of the stomach.

COMMENTS

Background

Endoscopic submucosal dissection (ESD) is an excellent treatment for early gastric cancer, but bleeding after ESD occurs in up to 7% of cases and remains a severe complication.

Research frontiers

Prophylactic coagulation of visible vessels in the resection area has been reported to reduce the bleeding rate, but preventive coagulation therapy cannot fully solve the problem of delayed bleeding. Thus, an assessment of risk factors for post-ESD bleeding is needed. The authors reviewed their 398 ESD cases and evaluated risk factors for post-ESD bleeding.

Innovations and breakthroughs

Beginner coagulators, tumor in the lower third of the stomach, and medicines potentially related to gastric injury/bleeding were significant risk factors for post-ESD bleeding. Bleeding at the ulcer margin occurred more frequently with beginner coagulators than with experts. Therefore, novice ESD operators should pay special attention to coagulating vessels in the ulcer edge after ESD. As to tumors in the lower third of the stomach, post-ESD bleeding happened earlier than in other areas. A second-look endoscopy 1 d after ESD may be helpful.

Applications

By overcoming post-ESD bleeding, ESD for gastric neoplasms will become a safer and more popular treatment.

Terminology

In this paper, experts/beginners were defined as endoscopists that have performed more than 200/less than 50 cases of gastric ESD.

Peer review

This is well written and important paper. Although it is not a prospective study, the topic is important for all gastroenterologists and gastrointestinal (GI) surgeons dealing with surgical endoscopy of the upper GI tract. The authors' work gives important information about one of the typical complications i.e. bleeding after ESD.

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BRIEF ARTICLE

Shear wave velocity is a useful marker for managing nonalcoholic steatohepatitis

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Abstract

AIM: To investigate whether a noninvasive measurement of tissue strain has a potential usefulness for management of nonalcoholic steatohepatitis (NASH).

METHODS: In total 26 patients, 23 NASHs and 3 normal controls were enrolled in this study. NASH was staged based on Brunt criterion. At a region of interest (ROI), a shear wave was evoked by implementing an acoustic radiation force impulse (ARFI), and the propagation velocity was quantified.

RESULTS: Shear wave velocity (SWV) could be reproducibly quantified at all ROIs in all subjects except for 4 NASH cases, in which a reliable SWV value was not calculated at several ROIs. An average SWV of 1.34 ± 0.26 m/s in fibrous stage 0-1 was significantly slower than 2.20 ± 0.74 m/s and 2.90 ± 1.01 m/s in stages 3 and 4, respectively, but was not significantly different from 1.79 ± 0.78 m/s in stage 2. When a cutoff value was set at 1.47 m/s, receiver operating characteristic analysis showed significance to dissociate stages 3 and 4 from stage 0-1 (P = 0.0092) with sensitivity, specificity and area under curve of 100%, 75% and 94.2%, respectively. In addition, the correlation between SWV and hyaluronic acid was significant (P < 0.0001), while a tendency toward negative correlation was observed with serum albumin (P = 0.053).

CONCLUSION: The clinical implementation of ARFI provides noninvasive repeated evaluations of liver stiffness at an arbitrary position, which has the potential to shed new light on NASH management.

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Key words: Nonalcoholic steatohepatitis; Ultrasound; Liver stiffness measurement; Shear wave velocity; Acoustic radiation force impulse

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INTRODUCTION

Nonalcoholic fatty liver diseases (NAFLD) are a pandemic over the globe, especially in the developed countries depending on a high calorie diet and sedentary life style. Nonalcoholic steatohepatitis (NASH) is a subgroup of NAFLD, in which chronic necroinflammatory response causes fiber accumulation in the liver[1]. Regardless of the etiology, accumulation of fiber in the liver is correlated with a deterioration of functional liver reserve. By semi-quantifying the density of fibrosis in the liver, histological evaluation is a standard strategy used to confirm the presence of chronic necroinflammation, and to evaluate a degree of the pathophysiological extent of liver damage[2]. Unfortunately, however, it is impractical to conduct liver biopsy in a large population of NAFLD and to repeat it through a course of NASH.

Several markers have been reported to be useful for the prediction of liver fibrosis, which can be categorized into three groups. One is a physical marker such as FibroScan®: transient elastography (TE)[3] and a digital quantification using contrast magnetic resonance imaging (MRI)[4]. The others are serum markers and can be further divided into two groups; direct and indirect markers. Serum markers are affected in both the inside and outside of the liver, while MRI may not be affordable for a large NAFLD cohort. TE is reported to be inaccurate for cases with higher body mass index (BMI) and hepatic steatosis[5].

Acoustic radiation force is a method exploiting energy in transmitted acoustic pulses to deliver a localized, precise force sufficient in magnitude to induce subtle tissue displacements[6]. The induced displacements can be tracked using conventional ultrasonic means and are indicative of local tissue mechanical properties[7]. Acoustic radiation force-based methods have been demonstrated for differentiating tissue structure *via* a mechanical property in various applications including the liver[8]. Here we report quantification of shear wave velocity (SWV) comparing with histology and serum biomarkers in NASH cases under ultrasound imaging coupled with the first clinical implementation of acoustic radiation force impulse (ARFI). The usefulness and limitations of SWV measurement are discussed.

MATERIALS AND METHODS

Patients

Twenty six cases, consisting of 23 patients suffering from NASH and 3 normal controls, were enrolled in this study. NASH was diagnosed when each of the following requirements were met; (1) abnormal values of alanine aminotransferase; (2) no positive results for hepatitis B surface antigen (HBsAg), anti-hepatitis C virus (anti-HCV), and anti-mitochondrial antibody; (3) histological findings consistent with NASH; and (4) neither suspicious drug usage, alcohol abuse, hereditary diseases nor any other clinical manifestations causing liver cell damage. Three normal controls were patients with each of chronic cholecystitis, chronic pancreatitis, and gallbladder polyps, who fulfilled all requirements for NASH diagnosis except for abnormal values of the transaminase and histological abnormalities, which were not evaluated. Two expert pathologists independently evaluated liver biopsy specimens and judged fibrous staging and inflammatory grading on the basis of Brunt criterion[9]. Informed consent was obtained from each patient, and the study protocol conformed to ethical guidelines of the 2008 Declaration of Helsinki as reflected in a prior approval by Niigata University Graduate School of Medical and Dental Sciences Human Research Committee.

Biochemical analyses

HBsAg and anti-HCV antibody were detected by a chemiluminescence immunoassay using ARCHITECT HBsAg QT and ARCHITECT HCV (Abbott Japan Co. Ltd., Chiba, Japan), respectively. Anti-mitochondrial antibody was quantified in sera using commercial kits of AMA; FluoroAID-1 test (MBL Co. Ltd., Nagoya, Japan). Hyaluronic acid was measured using a latex agglutination method, in which normal controls measure less than 50 ng/mL.

Measurement of shear wave velocity

SWV was measured using an ACUSON S2000 ultrasound system (SIEMENS Medical Solutions Inc., CA, USA). A single ultrasonic transducer array is used to both apply temporally short localized radiation forces within tissue and to track the resulting tissue dynamic response through time. During a regular ultrasound observation, region-of-interest (ROI) is set as a rectangle, and acoustic pulses are radiated to make tissues displace downward beside ROI. Once tissues are displaced, the shear stress starts to restore the tissue to its previous position and generate shear waves, which travel perpendicular to the direction of the acoustic push pulse in the process. The machine continuously monitors tissue positions in ROI and automatically calculates the speed of the wave in m/s unit. Fundamentally, SWV can be measured at any portion in the liver as long as the ROI can be placed under ultrasound imaging except for a position where the acoustic pulse cannot effectively displace tissues, such as deeper than 5 cm from a probe, or inside a vasculature, cyst or gallbladder. Using an ACUSON S2000, one can switch from regular ultrasound imaging to SWV measurements seamlessly and repeatedly with neither special preparation nor technique.

Statistical analysis

Categorical data was compared using ANOVA analysis among four fibrosis stages. Fisher's exact test was em-

ployed to compare the frequency in a contingency table of each factor consisting of BMI-Age-Alanine aminotransferase (ALT)-Triglyceride (TG) (BAAT) score[10]. Correlations between SWV and variable parameters were quantified by calculating the Spearman correlation coefficient. In order to judge the clinical usefulness of fibrosis indicators, an area under a receiver operating characteristic curve (AUROC) was calculated. All analyses were performed using GraphPad Prism 4 software (GraphPad Software, Inc. La Jolla, USA), and a two-sided *P*-value less than 0.05 was considered statistically significant.

RESULTS

Shear wave velocity is reproducible at the measurement site but is not constant throughout the entire liver

Aiming to evaluate reliability in SWV measurement, ROI was placed at each of the right and left lobes, and SWV was independently measured three times at the same lobe in four patients with fibrosis stage 3, cases 10 to 13. SWV was reproducibly determined at each ROI as the average coefficient of variance was 11.9% ± 8.0% (Figure 1). The speeds, however, largely varied between right and left lobes. These results strongly indicate that a repeated measurement may not be necessary at the same ROI for compensation of an implementation error, but is required by applying various ROIs throughout the liver in order to evaluate an average rigidity of a whole liver.

Shear wave velocity is getting faster along progression of fibrosis stage

According to inconsistent SWVs between the left and right lobes, SWV measurements were performed by placing four ROIs, which were located in each of the four segments; right posterior, right anterior, left medial and left lateral segments. Among the 23 NASH cases, a reliable SWV could not be calculated in 4 cases; listed as cases 23 to 26 in Table 1. Case 24 carried massive ascites and the others had a BMI of over 30 kg/m^2. In terms of BMI, SWV could be measured in all cases with BMI less than 30 kg/m^2, whereas SWV could not be measured in 3 out of 10 cases with BMI of more than 30 kg/m^2. None of the ROIs that could not return a reliable SWV value were located in the right segments, but in the left. The 4 cases missing at least one of four SWV values were excluded from further analyses.

An average of 4 SWVs in each case was compared among the fibrosis stages. When controls were designated as a stage 0, SWV was significantly correlated with fibrosis stages ($P = 0.0018$, $r = 0.63$). As only one case with fibrosis stage 1 was enrolled, 3 controls were combined with stage 1 and subjected to further analysis as one group of stage 0-1. The velocities in stages 0-1, 2, 3, and 4 were 1.34 ± 0.26, 1.79 ± 0.78, 2.20 ± 0.74, and 2.90 ± 1.01, respectively, and were significantly different among groups (Figure 2A, $P = 0.043$). Dunn's multiple comparison revealed that there was a significant difference between stages 0-1 and 4 ($P < 0.05$). When the velocity was compared in the right or left lobe, there was no significant difference in the right lobe ($P = 0.31$, Figure 2C), but there was a difference in the left lobe ($P = 0.02$, Figure 2D), in which SWVs were significantly different not only between stages 0-1 and 4, but also between 0-1 and 3 ($P < 0.05$, $P < 0.05$, respectively). There was no significant difference in SWV among inflammation activity grades ($P = 0.12$).

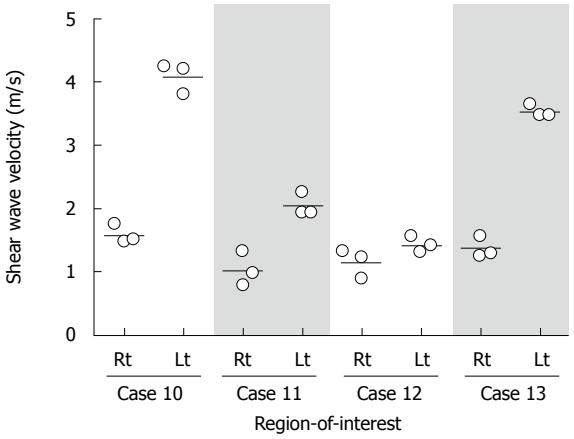


Figure 1 Reliability and variation of shear wave velocity (SWV) throughout the liver. SWV was measured independently three times at each of the right (Rt) or left (Lt) lobes in four patients with fibrosis stage 3, labeled cases 10 to 13. Each horizontal bar indicates an average SWV.

Shear wave velocity can identify fiber accumulation with high probability

Because it was suggested that SWV has a potential to discriminate fibrosis stage 3 or more from stage 0-1, a probability of the discrimination was evaluated using ROC analysis. SWV showed sensitivity and specificity of 100% and 75%, respectively when a speed of 1.47 m/s was selected as a cut-off value leading to a significant AUROC of 94.2% (Figure 3A, $P = 0.0092$). In contrast, platelet counts did not lead to a significant AUROC to distinguish stages 3 and 4 from 0-1 in our population (Figure 3B, $P = 0.52$, AUROC = 61%). Because several combinatorial systems were reported for identifying liver fibrosis in NAFLD, BAAT[10] and NAFLD fibrosis score[11] were calculated (Table 1). In the results, AUROCs of BAAT and NAFLD fibrosis score were 76.9% and 72.1%, respectively, and neither of these systems were significant in this setting ($P = 0.16$ and $P = 0.18$, respectively). Furthermore, none of the factors making up BAAT score; BMI, age, ALT and TG, showed a different frequency between fibrosis stage 0-1 and stages 3 and 4 ($P = 1.0$, 0.26, 1.0 or 1.0, respectively).

Shear wave velocity is correlated with markers not only for fibrosis but also for functional liver reserve

Next, a correlation was analyzed between SWV and serum markers. Hyaluronic acid was measured in sixteen out of nineteen NASH cases and showed a tight positive correlation with SWV (Figure 4A, $P < 0.0001$, $r = 0.84$). In addition, albumin concentration in the serum tended to be correlated with SWV in a negative fashion (Figure 4B, $P = 0.053$, $r = -0.42$). On the other hand, prothrombin time,

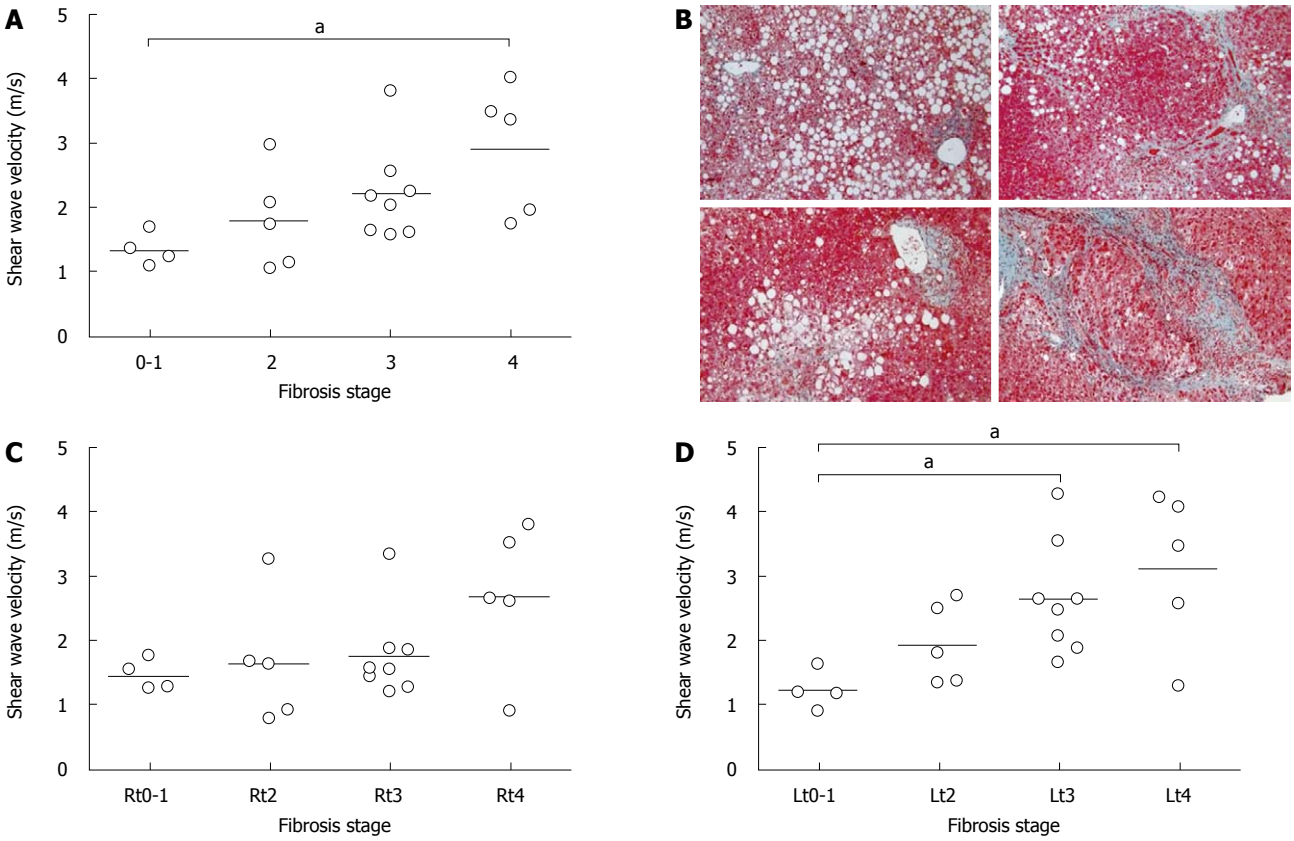


Figure 2 Average shear wave velocity (SWV) and representative photomicrographs in each fibrosis stage. SWV was measured in four segments; right anterior, right posterior, left medial and left lateral segments, and the average was calculated based on data from four segments (A) or from either one of right [Rt, (C)] or left [Lt, (D)] lobes. Histological fibrosis stages were judged along with Brunt criterion. Each horizontal bar indicates an average SWV. [a]$P < 0.05$. The photomicrographs (B) are representative Masson's trichrome stains for fibrosis stages 1, 2, 3 and 4 from upper left, lower left, and upper right to lower right panels, respectively. Original magnification is × 100.

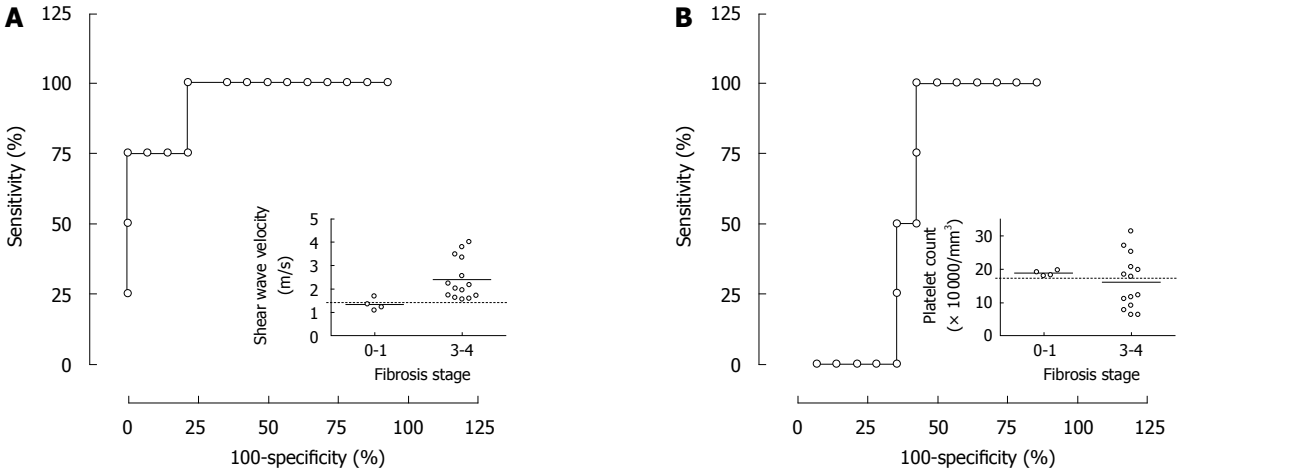


Figure 3 Receiver-operator characteristic curve (ROC) of SWV or platelet count to distinguish fibrosis stages 3 and 4 from stage 0-1. ROC to discriminate fibrosis stages 3 and 4 from stage 0-1 by SWV (A) or platelet count (B) revealed sensitivity, specificity and area under ROC of 100%, 75% and 94.2%, or 100%, 57.1% and 61%, respectively, when 1.47 m/s or 17.8 × 10 000/mm^3 was selected as a cutoff value. A small graph incorporated in each ROC graph shows the distribution of SWV or platelet count in fibrosis stages 0-1 and 3-4. The dotted line indicates the cut-off value selected.

age and BMI were not significantly associated with SWV ($P = 0.18$, $P = 0.42$ and $P = 0.46$, respectively).

DISCUSSION

Fundamentally, tissue displacements generated by acoustic push pulse used in ARFI are inversely related to tissue stiffness, while tissue recovery response is related to tissue viscoelastic properties[12]. In addition, SWV is positively correlated with the major mechanical properties indicating material rigidity[13]. Thus, assuming the liver is a linear, isotropic, elastic body, SWV is an indicative factor of tissue

Table 1 Case summary

No.	Diagnosis	Age (yr)	Gender	BMI (kg/m^2)	Stage[1]	Grade[1]	SWV (m/s)	ALB (g/dL)	AST (IU/L)	ALT (IU/L)	TG (mmol/L)	Plt (× 10^4/mm^3)	PT (%)	HA (ng/mL)	BAAT	NFS
1	Chr. cholecystitis	73	F	20.8			1.08	4.4	22	21	1.31	17.9			1	-1.2
2	Chr. pancreatitis	77	M	16.9			1.23	4.4	22	10	0.43	18.3			1	-0.3
3	Gall bladder polyp	80	M	17.1			1.36	4.0	21	20	0.72	19.7			1	-1.3
4	NASH	14	M	22.2	1	1	1.69	4.9	102	255	1.74	19.1	100	11	2	-4.4
5	NASH	18	M	35.1	2	3	1.73	4.9	46	84	1.83	24.0	104	13	3	-3.5
6	NASH	61	F	27.7	2	2	2.97	4.3	61	70	0.93	20.6	104	161	2	-1.5
7	NASH	41	F	40.8	2	3	1.05	4.6	83	156	1.43	24.9	100	11	2	-2.1
8	NASH	15	M	27.9	2	2	1.14	5.4	95	185	0.93	27.2	116	10	1	-4.0
9	NASH	39	F	20.8	2	3	2.06	3.3	72	28	4.17	17.1	75	29	1	-0.1
10	NASH	53	M	26.6	3	2	2.55	4.3	91	129	2.27	25.3	108	102	3	-1.5
11	NASH	51	F	26.6	3	3	1.57	4.3	49	75	1.78	31.4	126	60	3	-2.4
12	NASH	56	F	30.5	3	2	1.63	4.2	53	65	0.89	9.1	82	34	3	0.1
13	NASH	43	M	31.6	3	1	2.24	4.4	36	33	1.26	7.8	87	92	1	1.2
14	NASH	20	M	22.2	3	3	2.03	4.7	156	243	2.57	17.7	74	17	2	-3.6
15	NASH	46	M	31.1	3	2	3.80	4.6	28	26	2.46	20.6	100	412	2	-0.6
16	NASH	66	F	24.1	3	3	2.17	3.9	128	68	1.22	19.8	97	231	2	-0.3
17	NASH	11	M	21.8	3	3	1.60	4.7	39	68	0.86	27.1	95	14	0	-5.3
18	NASH	75	F	34.0	4	1	4.01	3.7	43	40	1.39	11.7	86	NT	2	2.5
19	NASH	82	M	23.9	4	2	3.35	3.7	67	48	0.83	12.3	72	259	1	2.1
20	NASH	70	F	22.1	4	2	1.95	4.7	44	33	1.60	18.4	79	92	1	-0.1
21	NASH	71	F	32.0	4	2	3.48	2.9	33	21	0.78	11.2	62	NT	2	3.3
22	NASH	71	M	27.0	4	2	1.73	2.9	36	22	0.73	6.4	59	NT	1	2.4
23	NASH	74	F	31.1	4											
24	NASH	75	F	24.1	4											
25	NASH	73	F	30.2	3											
26	NASH	17	M	37.8	3											

[1]Fibrosis stage and inflammation grade in liver biopsy specimen based on Brunt criterion. NASH: Nonalcoholic steatohepatitis; BMI: Body mass index; SWV: Shear wave velocity; ALB: Albumin; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; TG: Triclyceride; Plt: Platelet count; PT: Prothrombin time; HA: Hyaluronic acid; NT: Not tested; BAAT: BMI, age, ALT and TG score; NFS: NAFLD (nonalcohlic fatty liver diseases) fibrosis score.

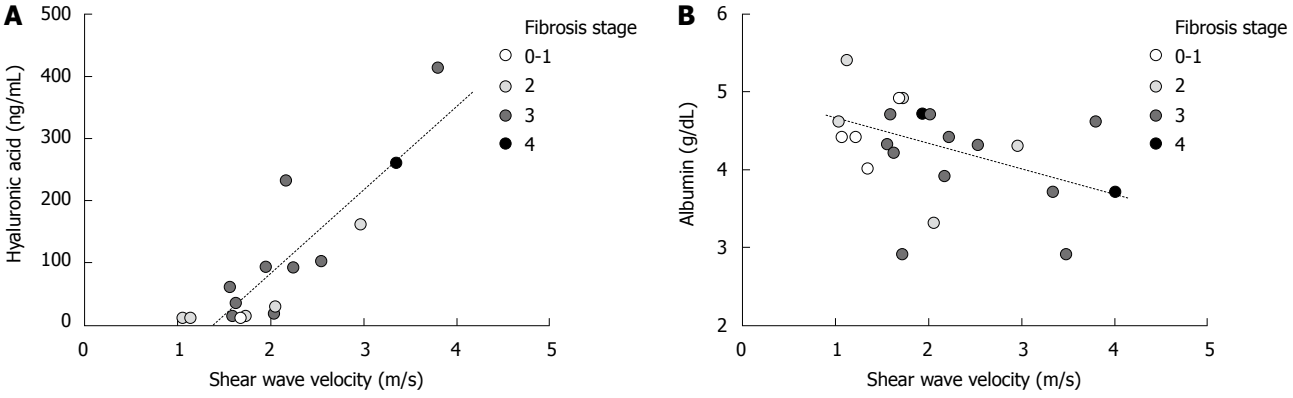


Figure 4 Correlation between SWV and serum biochemical parameters. SWV was plotted against hyaluronic acid (A) and serum concentration of albumin (B). A gray scale indicates the fibrosis stages, with white representing 0-1 through to dark gray for stage 4.

rigidity. Theoretically, the stiffer the tissue, the faster the shear wave will be propagated. Consistently, in this study SWV was correlated with histological progression of fibrosis and representative serum markers for fibrosis such as hyaluronic acid and collagen type Ⅳ (data not shown) as well. Although ARFI imaging has been used for delineating tissue structure *via* mechanical properties in numerous applications including chronic liver diseases[14,15], this is the first report to quantify SWV by implementing ARFI in the clinic and show a significant correlation with fiber deposition focusing on the NASH liver.

As a noninvasive methodology to quantify tissue stiffness in the liver, TE had been applied in the clinic[16]. TE mounts an ultrasound transducer on the axis of a vibrator, which transmits vibrations to propagate elastic shear waves. The major differences between TE and ARFI implemented SWV measurement are a real-time imaging capability, target size and target position. TE utilizes a vibration at audibility range and targets tissues without imaging supports as a 10 mm wide and 40 mm long cylinder, which is required to lie between 25 and 65 mm below the skin and to be free from large vascular structures. Given

those requirements, the area available for TE is limited to a portion of right lobe, and practically it is difficult to compare the stiffness at different parts of the liver. On the other hand, S2000 radiates the push pulse at MHz order to excite tissue displacement and scrutinizes SWV at ROI, which is substantially smaller (7 mm × 10 mm) and can be precisely placed at any position throughout the liver under real-time image guidance as long as it is not deeper than 5 cm from the probe. Because the interface evokes ultrasound-guided liver biopsy, "acoustic biopsy" may be coined for this technology. The major advantage of acoustic biopsy is the feasibility to be able to perform as many in time and space without a risk to patients. In this study SWV measurements were performed in four different segments at the same time point and revealed that SWV largely varied in different portions of the liver, while reproducible values were obtained at each ROI. Although the small number of subjects employed in this study limited the implication, these data support the idea that fibrogenesis and/or fibrolysis heterogeneously take place in the liver[17]. It must give us critical insight into the process of liver fibrosis to take acoustic biopsies from a wide area of the liver at different time points.

In terms of measurement failures, it was reported that TE frequently failed to compute liver stiffness in patients with massive ascites or BMI over 28 kg/m^2[5], and SWV measurements showed a similar tendency. One hypothesis is that the tissue displacement cannot be efficiently induced by radiation of acoustic push pulse through massive ascites or dense subcutaneous fat layers. However, it is noteworthy that SWV could be measured in right lobes of the four failed cases. All ROIs that returned ineffective SWV were located in left lobes. Although the exact reasons are not clear, oscillation of the liver due to heart beat may be an important factor to hamper accurate measurements. It has been shown that SWV in stiffer livers is associated with larger variability and smaller overall displacements[8], which suggest susceptibility of this technology to motion artifacts. A prospective study is required to clarify whether SWV in right lobes can be a useful indicator for liver fibrosis in those cases.

As for variability of SWV between right and left lobes, a considerable sampling variability was reported in liver biopsy specimens from patients with chronic hepatic diseases[18]. The concordance between two biopsy specimens that were simultaneously obtained from the same individual was reported to be moderate to low in terms of perisinusoidal fibrosis and other histological pathologic features of NAFLD[19]. The interlobar difference of SWV that was observed in this study seems to be consistent with the sampling variability suggesting nonhomogenous progression of NAFLD. Because sequential biopsies from multiple sites are practically impossible, longitudinal measurements of SWV at numerous locations of the liver in a large NAFLD cohort would be able to elucidate distribution of the pathological involvement in time and space.

Evaluation of liver fibrosis is aimed at two aspects of NASH management; one as a diagnostic tool and the other as a scrutiny of disease progression. Although histopathological examination is the current gold standard for evaluation of liver fibrosis, there are serious concerns for this strategy such as its invasive nature, sampling errors and diagnostic variability among observers[2]. To circumvent these limitations, surrogate serum markers have been reported such as BAAT score[10], NAFLD fibrosis score[11], hyaluronic acid[20], platelet count[21], and NashTest[22]. TE and MRI were also reported to show satisfying sensitivity and specificity to surrogate histological evaluation[4,16]. All noninvasive methodologies, however, have been shown to be effective in predicting advanced diseases rather than fibrogenic evolution, which is a hallmark to distinguish NASH from other NAFLD. Furthermore, it is impractical to perform an MRI study on a great number of NAFLD patients. Because SWV was not significantly different between fibrosis stages 0-1 and 2, and because the cohort in this study did not include NAFLD cases without fiber accumulation, the significance of SWV measurement in NASH diagnosis is also unclear. A further analysis should be conducted on a larger sample group including NAFLD cases without liver fibrosis to elucidate this issue.

As far as disease progression is concerned, platelet count, which has been confirmed as a useful surrogate marker for fibrosis in chronic hepatitis C[23], did not show significant AUROC in our cohort. A regulation of platelet count may be different between NASH and chronic hepatitis C. Although the NAFLD fibrosis score clearly distinguished fibrosis stage 4 from stages 0-1 (data not shown), AUROC was not able to distinguish stages 3 and 4 from stage 0-1, and there was no significant correlation between progression of liver fibrosis and the score, either. In BAAT, neither the score nor each factor used to calculate the score could discriminate fibrosis stages 0-1 from stages 3 and 4. Values of serum markers can be affected by extra hepatic conditions and/or a type of background diseases[24]. Furthermore, fibrosis markers have to be investigated over time to see pathophysiological progression. Serum markers at different time points would be affected by difference of systemic situation. Thus, it is most suspicious that the different populations among studies might cause different discrimination abilities in those indirect serum markers. As for the NashTest, it requires 13 parameters including α2-macroglobulin, apolipoprotein A1, and haptoglobin, which are not covered by public health insurance in Japan for patients with liver dysfunction, thus it is not affordable for significant numbers of patients. On the other hand, SWV tended to get faster along the progression of fibrosis in a stepwise fashion. Because a standard histopathological analysis has the same difficulty in discriminating adjacent stages of fibrosis[18], the usefulness in stepwise discrimination should be evaluated by a longitudinal study in a larger cohort.

There are a few reports that mention liver stiffness in association with biochemical properties of functional

liver reserve. Kawamoto *et al*[25] revealed positive correlations of TE with prothrombin time, platelet count and pre-albumin. Masuzaki *et al*[26] also reported a tendency of positive correlations with prothrombin activity, platelet count, and albumin concentration. In both reports the subjects were patients with chronic viral hepatitis type B and/or C. Interestingly, in our NASH cohort, prothrombin activity and platelet count were not significantly correlated with SWV, whereas serum albumin showed a tendency towards negative correlation. Although it is not difficult to suppose that TE/SWV and the direct markers for fibrosis will show positive correlation regardless of viral hepatitis or NASH, as in the case of hyaluronic acid, the indirect markers would be regulated in a different way depending on the disease responsible. The association between SWV and biochemical properties may implicate a significance of SWV measurement as a prognostic indicator.

The substitution of histopathological analysis of liver biopsy with a surrogate noninvasive marker would have a tremendous impact on both public health and health economics. Our data suggest that SWV measurements in implementation of ARFI have considerable potential to confirm or to exclude cirrhosis. Furthermore, this technology provides a repeated data acquisition for tissue mechanical properties from precise points throughout the liver. The actual longitudinal evaluation of fibrosis at different areas over the liver in a larger NAFLD population may prove not only useful for staging of NASH but also the process of fiber accumulation and value as a prognostic indicator.

COMMENTS

Background

Nonalcoholic steatohepatitis (NASH) is manifestation of metabolic syndrome in the liver and is a pandemic over the globe especially in the developed countries, based on a high calorie diet and sedentary life style. As in the other types of chronic hepatitis, collagen fibers continuously accumulate in the liver through the course of NASH toward cirrhosis and hepatocellular carcinoma development. Histological evaluation is a current gold standard for quantification of the fiber deposition. A tiny biopsy specimen, however, leads to considerable variability and is practically difficult to obtain repeatedly from multiple sites. Several surrogate markers have been developed for evaluation of fiber accumulation in a noninvasive way. Unfortunately, however, so far no methodology can satisfy both specificity for the liver and applicability for multiple sites.

Research frontiers

The significance of serum surrogate markers for histological evaluation of fibrosis has been reported, with examples including BMI-Age-Alanine aminotransferase-Triglyceride score, nonalcoholic fatty liver diseases (NAFLD) fibrosis score, hyaluronic acid, platelet count, and NashTest. Transient elastography and magnetic resonance imaging were also reported to show satisfying sensitivity and specificity to surrogate the histological evaluation. None of the serum markers, however, are specific for the liver but are affected by systemic involvement. Transient elastography requires a relatively large area to measure tissue rigidity, which prevents multifocal measurements. Magnetic resonance imaging is too laborious and expensive to target a large NAFLD cohort.

Innovations and breakthroughs

Using ACUSON S2000 ultrasound system, the research team led by Suda T from Department of Gastroenterology and Hepatology of Niigata University investigated the efficacy of shear wave velocity (SWV) measurement in the evaluation of NASH pathophysiology. ACUSON S2000 is the world's first ultrasound system implementing acoustic radiation force impulse to enable SWV measurement in the liver. The analyses revealed significant positive correlation between SWV and the histological fibrous stages and suggested the possibility to distinguish mild fibrosis from severe fibrosis or cirrhosis with substantial accuracy. Furthermore, the correlations of SWV with biochemical markers such as hyaluronic acid or albumin were presented. This is the first report suggesting that SWV can be reproducibly measured in NASH and varies through the liver.

Applications

Due to the non-invasive and easily accessible nature of SWV measurement, this technology makes it possible to conduct a longitudinal evaluation of liver rigidity at multiple sites of the liver in a large cohort. The actual longitudinal evaluation in a larger NAFLD population may prove not only useful for fibrous staging of NASH but also for the process of fiber accumulation and value as a prognostic indicator.

Terminology

SWV is the speed of a transverse wave propagating perpendicular to the direction of tissue displacement. Assuming the liver is a linear, isotropic, elastic body, SWV is an indicative factor of tissue rigidity. Theoretically, the stiffer the liver, the faster the shear wave will be propagated. Acoustic radiation force impulse is a phenomenon due to energy transfer associated with acoustic frequency higher than tissue attenuation capability. The energy results in a momentum transfer generating a force that causes tissue displacement.

Peer review

This is a well-conducted study that evaluates the usefulness of SWV in quantifying liver fibrosis in patients with NASH.

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BRIEF ARTICLE

Dual specimens increase the diagnostic accuracy and reduce the reaction duration of rapid urease test

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Abstract

AIM: To evaluate the influence of multiple samplings during esophagogastroduodenoscopy (EGD) on the accuracy of the rapid urease test, and the validity of newly developed rapid urease tests, HelicotecUT plus test and HelicotecUT test, CLO test and ProntoDry test.

METHODS: A total of 355 patients undergoing EGD for dyspepsia were included. Their *Helicobacter pylori* (*H. pylori*) treatment status was either naïve or eradicated. Six biopsy specimens from antrum and gastric body, respectively, were obtained during EGD. Single antral specimens and dual (antrum + body) specimens were compared. Infection status of *H. pylori* was evaluated by three different tests: culture, histology, and four different commercially available rapid urease tests (RUTs)-including the newly developed HelicotecUT plus test and HelicotecUT test, and established CLO test and ProntoDry test. *H. pylori* status was defined as positive when the culture was positive or if there were concordant positive results among histology, CLO test and ProntoDry test.

RESULTS: When dual specimens were applied, sensitivity was enhanced and RUT reaction time was significantly reduced, regardless of their treatment status. Thirty minutes were enough to achieve an agreeable positive rate in all the RUTs. Both newly developed RUTs showed comparable sensitivity, specificity and accuracy to the established RUTs, regardless of patient treatment status, RUT reaction duration, and EGD biopsy sites.

CONCLUSION: Combination of antrum and body biopsy specimens greatly enhances the sensitivity of rapid urease test and reduces the reaction duration to 30 min.

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Key words: Rapid urease test; *Helicobacter pylori* eradication; HelicotecUT plus test; HelicotecUT test; Esophagogastroduodenoscopy

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INTRODUCTION

Since the discovery of *Helicobacter pylori* (*H. pylori*) by Marshall and Warren in 1983[1], overwhelming evidence confirms that *H. pylori* infection plays a significant role in the development of chronic active gastritis, peptic ulcer, and even gastric adenocarcinoma[2-4]. *H. pylori* infection is very common throughout the world, occurring in 40%-50% of the population in developed countries, 80%-90% in developing regions[5], and 54.4% in Taiwan[6]. The treatment of gastrointestinal (GI) disorders resulting from *H. pylori* infection has become the leading issue in clinical practice.

A large number of methods have been utilized to diagnose the status of *H. pylori* infection, and most of them require endoscopic biopsies for tissue sampling. Nonetheless, to date, no single test other than culture is able to confirm *H. pylori* infection[7-9]. There are inherent advantages and disadvantages in each diagnostic test. For example, rapid urease tests (RUTs) are subject to false-negative results due to sampling errors. After *H. pylori* eradication, bacterial load may be reduced to patchy colonization, which is likely to result in false negative findings[10]. This prompts us to reappraise the feasibility of current sampling methods. One small piece of gastric mucosa specimen sampled from the antrum is the most popular method for RUT. A strategy for multiple samplings is required to improve the diagnostic yield. In spite of this, RUT is regarded of one of the best tests for diagnosis of *H. pylori* infection, given its high sensitivity and specificity. In addition, RUT is user friendly and cost effective[11-14]. Whereas some commercial kits may require at least 4 h[15,16], the ideal RUT reaction duration is 30 min because it enables endoscopists to obtain the results before patients leave the endoscopy suite.

The aim of this prospective study is to evaluate the validity of newly developed rapid urease tests, HelicotecUT plus test and HelicotecUT test, and the influence of multiple samplings during esophagogastroduodenoscopy (EGD) on the accuracy of the rapid urease test.

MATERIALS AND METHODS

Subjects

Between December 2008 and August 2009 355 patients in Kaohsiung Medical University Hospital, Kaohsiung, Taiwan were included in this study. They were scheduled to undergo EGD to evaluate dyspepsia and exclude organic lesions because their symptoms failed to improve after medication. All patients signed their informed consent and received complete EGD. Patients were separated according to their *H. pylori* treatment status: 200 patients (82 men, 118 women; mean age: 55.9 years, range: 17-86 years) in the treatment naïve group and 155 patients (69 men, 86 women; mean age: 58.0 years, range: 26-83 years) in the eradicated group. Patients meeting the following criteria were excluded: antibiotics, bismuth salts, or proton pump inhibitor used during the previous 1 mo; prior gastric surgery; presence of a bleeding peptic ulcer; severe concomitant diseases; and pregnancy or lactation.

Diagnostic tests for H. pylori infection

Histological examination was done in the pathology core lab, and the gastric specimens were fixed with formalin, embedded in paraffin and stained with hematoxylin and eosin. Culture of *H. pylori* was carried out on the second set of specimens, which were rubbed on the surface of a Campy-BAP agar plate [Brucella agar (Difco) + IsoVitalex (Gibco) + 10% whole sheep blood], and then incubated at 35℃ under microaerobic conditions (5% O_2, 10% CO_2, and 85% N_2) for 4-5 d. *H. pylori* culture was considered as positive if one or more colonies of gram-negative, oxidase (+), catalase (+), and urease (+) spiral or curved rods were present. The results of RUT were interpreted as positive if the color of the gel changed from yellow to pink or red within 24 h at room temperature, and the reaction duration was recorded in minutes accordingly. A total of four RUTs were examined, including HelicotecUT plus test (Strong Biotech Corp-Taiwan), HelicotecUT test (Strong Biotech Corp-Taiwan), CLO test (Kimberly Clark-USA), and ProntoDry test (Medical Intruments Corp-France). The media contained in the CLO test and HelicotecUT were semi-solid phase (agar gel) and in HelicotecUT plus and ProntoDry test were solid phase (dry plate). Single antral specimens and dual (antrum + body) specimens were compared as well.

Confirmation of H. pylori infection

Six biopsy specimens from the lesser curvature side of the antrum and the greater curvature side of the gastric body, respectively were obtained to afford culture, histology and 4 different RUTs. The infection status of *H. pylori* infection was considered positive if the results from either antrum or gastric body met the following criteria: (1) positive culture; or (2) concordant positive results among histology and both CLO and ProntoDry tests. The results of both the CLO test and ProntoDry test were interpreted independently. After we confirmed *H. pylori* status, the diagnostic accuracy of each RUT was evaluated.

Table 1 Validity of rapid urease tests in the treatment naïve group

Method	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)	P^1
Sampling over antrum with reaction time: 30 min						
HelicotecUT plus	80.6	96.2	91.5	90.8	91.0	
HelicotecUT	77.6	98.5	96.3	90.0	92.0	
CLO	84.6	96.2	91.8	92.1	92.0	
ProntoDry	83.6	95.5	90.3	92.0	91.5	0.988
Sampling over antrum with reaction time: 24 h						
HelicotecUT plus	91.0	94.7	89.7	95.5	93.5	
HelicotecUT	85.1	94.7	89.1	92.6	91.5	
CLO	92.5	95.5	91.2	96.2	94.5	
ProntoDry	92.5	94.7	90.0	96.2	94.0	0.642
Sampling over antrum plus body with reaction time: 30 min						
HelicotecUT plus	98.5	92.5	86.8	99.2	94.5	
HelicotecUT	95.5	94.7	90.1	97.7	95.0	
CLO	97.0	94.0	89.0	98.4	95.0	
ProntoDry	97.0	91.7	85.5	98.4	93.5	0.902

[1]χ^2 test of four RUTs accuracy. RUTs: Rapid urease tests; PPV: Positive predictive value; NPV: Negative predictive value.

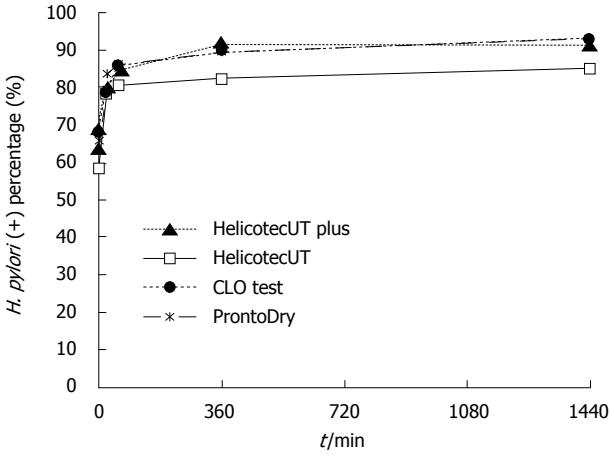


Figure 1 Detection rate and reaction time of four commercial rapid urease tests. *H. pylori*: *Helicobacter pylori*.

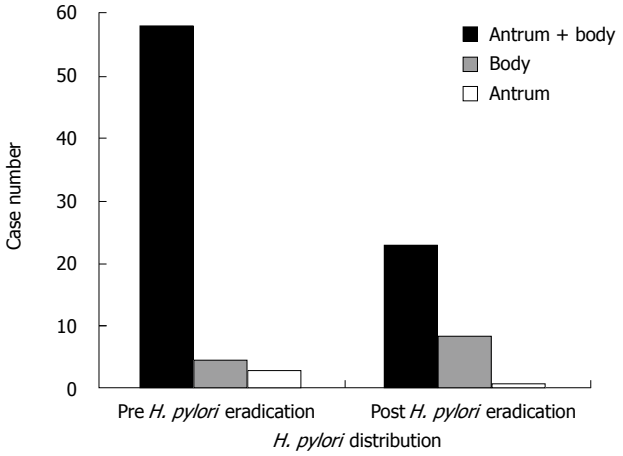


Figure 2 Distribution of *H. pylori* infection before and after eradication. The *P* value of χ^2 test was 0.03.

Statistical analysis

In this study, χ^2 test was chosen for statistical analysis.

RESULTS

The positive status of *H. pylori* infection was 33.5% (67/200) in the treatment naïve group and 21.9% (34/155) in the eradicated group. The culture yield in both groups was 67.2% (45/67) (21 men, 24 women; mean age: 57.8 years, range: 27-83 years) in the treatment naïve group and 44.1% (15/34) (6 men, 9 women; mean age: 55.0 years, range: 37-74 years) in the eradicated group. This result suggested the important role of other biopsy-based tests, particularly RUT, in establishing the diagnosis of *H. pylori* infection.

The diagnostic power including sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy of the four different RUTs (HelicotecUT plus test, HelicotecUT test, CLO test, ProntoDry test, presenting in the same order afterwards) was shown in Table 1. In the treatment naïve group, all four RUTs showed comparable sensitivities, specificities and accuracy at 30 min (P = 0.988) and at 24 h (P = 0.642). Although the diagnostic yield of *H. pylori* infection was slightly improved at 24 h, the tests appeared readable and accurate at 30 min (30 min *vs* 24 h, χ^2 test of accuracy, P = 0.156) (Figure 1). Interestingly, the diagnostic accuracy of dual specimens at 30 min was as comparable as single specimens at 24 h (χ^2 test of accuracy, P = 0.346).

In the eradicated group, the distribution of *H. pylori* shifted from antrum to gastric body after *H. pylori* eradication was clearly observed in this study (Figure 2). Because of the lower bacterial loads and patchy distribution, in all 4 RUTs, the diagnostic accuracy of single specimens at 30 min was as low as single specimens at 24 h (χ^2 test of accuracy, P = 0.124). Both results were significantly inferior to dual specimens at 30 min (χ^2 test of accuracy, P = 0.01) (Table 2).

DISCUSSION

There are many diagnostic tests available for *H. pylori* infection. Overall, patients prefer non-invasive tests over invasive tests. Consequently, recent studies have been dedicated to non-invasive tests as a result[17]. No statistical difference

Table 2 Validity of rapid urease tests in the eradicated group

Method	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)	P^1
Sampling over antrum with reaction time: 30 min						
HelicotecUT plus	50.0	100.0	100.0	87.7	89.0	
HelicotecUT	44.1	100.0	100.0	86.4	87.7	
CLO	55.9	100.0	100.0	89.0	90.3	
ProntoDry	50.0	100.0	100.0	87.7	89.0	0.913
Sampling over antrum with reaction time: 24 h						
HelicotecUT plus	61.8	100.0	100.0	90.3	91.6	
HelicotecUT	58.8	100.0	100.0	90.0	91.0	
CLO	64.7	99.2	95.7	91.0	91.6	
ProntoDry	64.7	100.0	100.0	91.0	92.3	0.983
Sampling over antrum plus body with reaction time: 30 min						
HelicotecUT plus	88.2	99.2	96.8	96.8	96.8	
HelicotecUT	82.4	100.0	100.0	95.3	96.1	
CLO	88.2	98.3	93.8	96.7	96.1	
ProntoDry	82.4	100.0	100.0	95.3	96.1	0.987

[1]χ^2 test of four RUTs accuracy.

was reported between these two kinds of tests, given their comparable diagnostic performances[14,18]. However, during the initial diagnosis of *H. pylori* infection, biopsy-based diagnostic tests are usually considered in terms of costs for patients who receive endoscopic examination. RUT is the method of choice among biopsy-based diagnostic tests given their user friendliness and cost-effectiveness[13,19,20]. Short reaction duration and high diagnostic performance are the two attributes of RUT. Therefore, RUT has gained its popularity among most gastroenterologists.

RUT is made available in many commercial kits. The CLO test has been established for more than a decade and used as the main standard for rapid urease tests. Its good performance, with a sensitivity of 89.6%, a specificity of 100%, a PPV of 100%, and a NPV of 84.1%, has been acclaimed[14]. Compared with the CLO test, the newly developed HelicotecUT plus test and HelicotecUT test are equally competent in terms of their diagnostic yield. Our data showed similar results among all four RUTs, even in the eradicated group. Given their comparable diagnostic performance, the tests of lower cost, like HelicotecUT plus test and HelicotecUT test, are of more advantage.

Sampling error is the major drawback of RUT. The diagnostic yield of biopsy-based methods is usually compromised in older patients who are more likely to have extensive gastric mucosa atrophy and intestinal metaplasia. The latter, if happening in the antrum, prevents *H. pylori* colonization and consequently promotes the migration of *H. pylori* to the gastric body. Several mechanisms have been suggested, including loss of a *H. pylori*-specific receptor-like structure on the epitherlium and active secretion of secretory IgA for metaplastic mucosa[21-23]. In addition, after *H. pylori* eradication, the suppression of the density and urease activity of *H. pylori* by antibiotics may reduce the sensitivity of RUT. Despite this, Rollán *et al*[24] reported good sensitivity in the CLO test (91.7%) in their study of 12 *H. pylori*-positive patients and 47 *H. pylori*-negative patients with duodenal ulcers after eradication treatment. They took 3 biopsy specimens from different sites and put them in one test vial. This suggested that more specimens may prevent the false negative results due to sampling error. In this study, we also found that dual specimens improved the overall sensitivity of RUT, even after eradication. Furthermore, dual specimens achieved the highest performance even at 30 min. Our results further portray that more specimens for diagnosis can amend the inherent error of biopsy-based tests.

The major limitation of this study is a lack of global evaluation tests, such as urea breathing test or stool *H. pylori* antigen test (HpsA), which have been established as reliable tests to detect the presence of *H. pylori* in the hosts. Sampling bias may exist within our biopsy-based diagnostic tests, including culture, histology and RUT. In addition, the prevalence of *H. pylori* infection was low in our study cohort. It may be that our patients were mainly from urban area (Kaohsiung City) and might have a lower *H. pylori* infection rate[6].

In conclusion, a combination of antrum and body biopsy specimens for rapid urease test greatly enhances sensitivity and reduces the reaction duration to 30 min. The newly developed HelicotecUT plus test and HelicotecUT test are as effective as the established CLO test and ProntoDry test.

COMMENTS

Background

Helicobacter pylori (*H. pylori*) infection plays a significant role in the development of chronic active gastritis, peptic ulcer, and even gastric adenocarcinoma. *H. pylori* infection has become the leading issue in clinical practice. A large number of methods have been utilized to diagnose *H. pylori* infection. Rapid urease test (RUT) is regarded of one of the best tests for diagnosis of *H. pylori* infection, given its high sensitivity and specificity. However, sampling bias and reaction were clinical issues in clinical practice of RUT.

Research frontiers

This study aimed to evaluate the validity of newly developed RUTs, HelicotecUT plus test and HelicotecUT test, and the influence of multiple samplings during esophagogastroduodenoscopy (EGD) on the accuracy of the RUT.

Innovations and breakthroughs

The newly developed HelicotecUT plus test and HelicotecUT test are as effective as the established CLO test and ProntoDry test.

Applications

Combination of antrum and body biopsy specimens for rapid urease test greatly enhances sensitivity and reduces the reaction duration to 30 min.

Peer review

In this manuscript, the authors compared the diagnostic value of two new rapid urease test kits for detection of *H. pylori* and they compared the diagnostic accuracy when analysing only one antral sample or when analysing a antral and corpal samples. The golden standard used to confirm *H. pylori* status was either a positive culture or concordant result between two to three other tests.

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BRIEF ARTICLE

Postoperative adjuvant antiviral therapy for hepatitis B/C virus-related hepatocellular carcinoma: A meta-analysis

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Abstract

AIM: To investigate the impact of postoperative antiviral treatment on tumor recurrence and survival of patients with chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infection-related primary hepatocellular carcinoma (HCC) after curative therapy.

METHODS: We performed a meta-analysis of randomized and non-randomized control trials from electronic search and manual search. The fixed effect model of Mantel-Haenszel method and the random effect model of Der Simonian and Laird method were used for homogeneous and heterogeneous studies, respectively. Seven HCV-related studies, three HBV-related studies and three studies on HBV or HCV-related HCC were identified.

RESULTS: A total of 1224 patients were included in this analysis. The estimated odds ratios (OR) for the 1-, 2-, 3- and 5-year recurrence were 0.54 [15.4% *vs* 24.1%, 95% confidence interval (CI): 0.32-0.89, P = 0.02], 0.42 (36.9% *vs* 58.0%, 95% CI: 0.19-0.90, P = 0.03), 0.37 (47.9% *vs* 63.8%, 95% CI: 0.19-0.71, P = 0.003), and 0.32 (66.7% *vs* 74.3%, 95% CI: 0.15-0.66, P = 0.002), respectively; and the OR for the 1-, 2-, 3-, 5- and 7-year mortality were 0.23 (1.2% *vs* 9.1%, 95% CI: 0.07-0.71, P = 0.01), 0.31 (6.4% *vs* 22.1%, 95% CI: 0.12-0.79, P = 0.01), 0.43 (12.7% *vs* 20.8%, 95% CI: 0.21-0.89, P = 0.02), 0.42 (25.1% *vs* 42.0%, 95% CI: 0.27-0.66, P = 0.0002) and 0.28 (31.9% *vs* 52.2%, 95% CI: 0.13-0.59, P = 0.0008).

CONCLUSION: This meta-analysis indicates the postoperative antiviral therapy, interferon in particular, may serve as a favorable alternative to reduce recurrence and mortality in patients with HBV/HCV related HCCs.

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Key words: Antiviral therapy; Hepatitis B virus; Hepatitis C virus; Hepatocellular carcinoma; Recurrence; Survival

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INTRODUCTION

Hepatocellular carcinoma (HCC) is common worldwide, particularly in Asia where chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infection is the most common etiology. Generally, hepatectomy, percutaneous ethanol injection (PEI), percutaneous microwave coagulation therapy (PMCT), radiofrequency ablation (RFA) and orthotopic liver transplantation are recognized as curative treatment options for HCC, although liver transplantation can only be offered to a small proportion of patients due to graft availability, selection criteria, and high cost. The long-term prognosis, however, remains guarded because of the high frequency of recurrence, which is also the main cause of death, in addition to concomitant hepatic decompensation.

Interferons (IFNs) are cytokines possessing a variety of biologic properties, including antiviral, immunomodulatory, antiproliferative, antiangiogenic and tumoricidal effects[1]. They are effective in suppressing the replication of HBV/HCV. Response to IFN treatment is associated with improved clinical outcomes and less cirrhosis-related complications. It is also reported that the combination of IFN and certain cytotoxic drugs has resulted in a modest response in patients with inoperable HCC[2]. Certain nucleoside/nucleotide analogues (NAs), such as lamivudine, adefovir, and entecavir, which inhibit the reverse transcriptase activity of viral DNA polymerase, have been demonstrated to be useful for HBV-infected patients, by means of not only suppressing the viral load but also reducing fibrosis in the liver[3,4]. Lamivudine treatment has been reported to consistently reduce HBV replication, and thus might improve the remnant liver function, prevent liver failure and prolong survival[5-7].

Up till now, although several randomized controlled trials (RCTs) and non-randomized controlled trials (NRCTs) have evaluated the efficacy and outcome of antiviral therapies in patients with virus-related HCC after curative treatment, no clear recommendations have been proposed yet. Usually, it is the small sample size of the RCTs that has resulted in a lack of power to detect clinically meaningful differences. In order to test the hypothesis that postoperative antiviral treatment would reduce or postpone recurrence and improve overall survival in patients with chronic HBV/HCV infection-related HCC after curative therapy, we performed a meta-analysis on the basis of published data from both RCTs and NRCTs in the literature.

MATERIALS AND METHODS

Criteria for inclusion and exclusion

Two investigators independently assessed the generated papers for relevance. In case of discrepancies found between the two investigators, a third investigator would make the definitive decision for study eligibility and data extraction.

Eligible patients were those who had undergone a curative treatment for primary HCC associated with HBV or HCV. Curative treatment was defined as complete tumor eradication, with no residual tumor visible by computed tomography, or resection of all evident tumor tissues, which included percutaneous tumor ablation [PTA, e.g. percutaneous ethanol injection (PEI), percutaneous acetic acid injection (PAI), radiofrequency ablation (RFA), microwave coagulation therapy (MCT), *etc.*], surgical treatment (e.g. hepatectomy, ablation during laparotomy, *etc.*), or their combinations. Antiviral therapy included regularly administered IFN (IFNα, IFNβ, and recombinant IFN) for HBV or HCV, nucleoside/nucleotide analogue such as lamivudine for HBV, and other anti-hepatitis virus medications. Primary outcomes were cumulative survival rate, mortality rate, and cumulative recurrence rate at 1, 2, 3, 5 or 7 years.

Studies with the following situations were excluded: (1) those evaluating antiviral therapies before primary HCC developed or after non-curative treatment or liver transplantation; (2) those evaluating an antiviral therapy in combination with chemotherapy; (3) those with no information on cumulative probability of survival, mortality or recurrence; and (4) those evaluating patients with other subtypes of liver malignancy, e.g. cholangiocellular carcinomas or liver metastases.

Literature search

We performed an electronic search of Medline, Embase, Cancerlit, and the Cochrane Library covering a period from 1980 to March 2009 to identify full publications or abstracts of literatures using the terms "hepatocellular carcinoma ", "hepatitis ", "recurrence", "survival", "antiviral agents", "interferon", "lamivudine", "adefovir", "entecavir" and "ribavirin".

In addition, manual search for the abstracts published in major international conferences were also conducted and general reviews and the reference lists from the identified publications were cross-checked to obtain other relevant publications.

Data collection and analysis

We considered articles presenting data from RCTs and NRCTs originally published in English. When the data of a study were included in more than one publication, we excluded studies with duplicated publications on the same group of patients, including those reporting the early stages of study findings with incomplete sample size; only the most recent and complete studies were included in the meta-analysis.

The intention-to-treat method was used. The measure of interest was the OR for cohort studies and the corresponding 95% CI. When ORs were not available in the published article, they were computed from the exposure distributions. For each meta-analysis, χ^2 test was used to assess the statistical heterogeneity among studies

Table 1 Characteristics of the included studies

RCT/ NRCT	Medium follow-up time (treatment, control) (mo)	HBV/HCV	Curative intervention	Regimen	Treatment	Control	Cumulative survival rate at year	Cumulative recurrence rate at year	SVR/ non-SVR	Ref.
NRCT	32, 31	HCV	Hepatectomy, RFA, PEI, PMCT, ablation under LC	IFNα 6 MIU Qd × 2 w + tiw × 22 w	42	42	3, 5, 7	1, 3, 5, 7	29/13	[22]
NRCT	37, 45	HCV	Hepatectomy, RFA, PEI, PMCT, ablation under LC	IFNα 3 MIU tiw × 48 w	16	16	1, 2, 3, 4	1, 2, 3	2/14	[23]
NRCT	NA	HCV	Hepatectomy, RFA, PEI, PMCT	IFNα 3-6 MIU tiw × 24-48 w	53	399	3, 6, 9	NA	17/36	[24]
NRCT	< 36	HCV	PEI, RFA, PMCT	IFNα-2b 3-5 MIU biw + ribavirin 1000-1200 mg Qd × 24-48 w	16	33	1, 2, 3	1, 2, 3	10/6	[25]
NRCT	28, 23	HCV	RFA	IFNα-2b 3 MIU biw × as long as possible	24	33	NA	1, 2, 3	1/23	[26]
RCT	85 ± 16	HCV	PEI	IFN 6 MIU tiw × 48 w	49	25	1, 3, 5, 7	1, 3, 5	14/35	[8]
RCT	25.0 (2.0-34.6)	HCV	Hepatectomy, PEI	IFNβ 6 MIU biw × 36 m	10	10	NA	1, 2	0/10	[27]
NRCT	38.0 (9.2-78.2), 32.6 (0.5-75.7)	HBV	Hepatectomy, RFA	lamivudine 100 mg Qd × as long as possible	16	33	1, 2, 3	1, 2, 3	NA	[28]
NRCT	53	HBV	Hepatectomy, ablation	IFNα biw/tiw × ≥ 24 w	11	69	NA	3, 5, 10	NA	[30]
NRCT	31	HBV	Hepatectomy, ablation	lamivudine 100 mg Qd	30	40	1, 2	1, 2	NA	[29]
RCT	30	HBV 77 HCV 2 HBV + HCV 1	Hepatectomy	IFNα-2b 10 MIU/m^2 tiw × 16 w	40	40	1, 3, 5	NA	NA	[16]
RCT	45	HCV 80 HBV + HCV 70	Hepatectomy	IFNα 3 MIU tiw × 48 w	76	74	3, 5	NA	2/74	[31]
RCT	27 (4-53)	HBV 16 HCV 13 HBV + HCV 1[1]	PAI	IFNα 3 MIU tiw × 24 w/ IFNα 3 MIU Qd × 10 d/m × 6 m + 10 d/3 m × 18 m	18	9	NA	1, 2, 3, 4	NA	[32]

[1]3 were excluded from the final analysis as they had already experienced one recurrence. NA: Not available; NRCT: Non-randomized controlled trial; RCT: Randomized controlled trial; HBV: Hepatitis B virus; HCV: Hepatitis C virus; IFN: Interferon; SVR: Sustained virologic response; RFA: Radiofrequency ablation; PEI: Percutaneous ethanol injection; PMCT: Percutaneous microwave coagulation therapy; PAI: Percutaneous acetic acid injection.

and results were defined as heterogeneous when *P* values were less than 0.10. A fixed effect model, the Mantel-Haenszel method, was used for homogeneous studies, and when significant heterogeneity existed, a random effect model, the Der Simonian and Laird method, would be applied (RevMan, version 5; The Cochrane Collaboration, Copenhagen, Denmark). A significance level of 5% was taken as the α risk. Only meta-analyses of antiviral therapies that included two or more studies (RCTs or NRCTs) were performed.

To compute summary OR for different viral etiology, we calculated the study-specific estimates separately for HBV and HCV. Forest plots were given. Publication bias was evaluated using funnel plots and Egger's test.

RESULTS

One hundred and twenty-four abstracts were found through the search in literature; among them, 17 studies with 22 publications fulfilled the inclusion criteria. Three additional publications from the bibliographies of retrieved studies were also identified. Among them, ten publications[8-17] reported five studies at different time, therefore, only the five most recent articles[8,10,12,16,17] were used for the analysis; five studies[12,17-20] did not report the number of death or recurrence at 1, 2, 3, 5 or 7 years or provide data separately for the treatment and control groups and were thereby excluded; one study[21] enrolled not only patients who received postoperative antiviral therapy but also those who were preoperatively treated, another study[10] included patients who received palliative treatment before antiviral therapy, both of which did not provide separate data on survival or recurrence for different treatment approaches and were therefore excluded. The final analysis included seven studies of HCV-associated HCC with IFN therapy[8,22-27], three studies of HBV-associated HCC, two with lamivudine[28,29] and one with IFN therapy[30], and three studies of HBV/HCV-associated HCC with IFN therapy[16,31,32] (Table 1).

Among the seven HCV-related studies using IFN, two were RCTs[8,27], the rest five were cohort studies (NRCTs)[22-26]. A total of 768 patients were enrolled, and 210 received IFN treatment. The medium duration of follow-up ranged from 23 to 85 mo. The three HBV-related studies are all NRCTs, with a total of 199 patients included; 11 patients received IFN treatment and 46 received lamivudine. The medium duration of follow-up ranged from 31 to 53 mo. All of the three HBV/HCV-related studies are RCTs with a total of 260 patients, including 93 HBV-infected, 95 HCV-infected, and 72 HBV/HCV-co-infected HCCs; three of them were excluded from the final analysis as they had already experienced one recurrence. Among the rest 257 patients, 134 received IFN therapy. The medium duration of follow-up ranged from 27 to 45 mo.

Antiviral therapy and HCC recurrence

For 1-year recurrence, pooled data from nine studies[8,22,23,25-29,32] showed significantly more HCC recurrence cases in the control (24.1%) than in the antiviral therapy (15.4%) groups (OR: 0.54, 95% CI: 0.32-0.89, P = 0.02, Figure 1A). No statistical heterogeneity was found (I^2 = 0%, P = 0.60). Subgroup analysis of the six HCV-related studies[8,22,25-27,32] revealed significantly fewer HCC recurrences in IFN groups (12.1%) than in the control groups (20.8%) (OR: 0.52, 95% CI: 0.27-1.00, P = 0.05) with no statistical heterogeneity (I^2 = 16%, P = 0.31). In the two HBV-associated studies[28,29], which were also the only two lamivudine-treated studies, no statistically significant difference was found between lamivudine (21.7%) and control groups (28.8%) (OR: 0.59, 95% CI: 0.24-1.43, P = 0.24). Subgroup analysis of the seven IFN studies, which combined outcomes of the six HCV-related and 1 HBV/HCV-related HCC studies[31], revealed that compared with control (22.0%), IFN groups had a lower HCC recurrence rate (13.7%) (OR: 0.52, 95% CI: 0.28-0.95, P = 0.03). No statistical heterogeneity was found (I^2 = 0%, P = 0.43).

For 2-year recurrence, as statistical heterogeneity (I^2 = 51%, P = 0.06) was found, the randomized model was applied to the data from seven studies[23,25-29,32], revealing significantly fewer HCC recurrences in the treatment (36.9%) than the control (58.0%) groups (OR: 0.42, 95% CI: 0.19-0.90, P = 0.03, Figure 1B). Subgroup analysis of the four HCV-related studies[23,25-27] showed significantly more HCC recurrences in the control (63.0%) than in IFN groups (30.3%) (OR: 0.23, 95% CI: 0.06-0.99, P = 0.05) with statistical heterogeneity (I^2 = 65%, P = 0.03). In the two HBV studies[28,29], no significant difference was found between lamivudine (47.8%) and control groups (50.7%) (OR: 0.82, 95% CI: 0.38-1.74, P = 0.60). Subgroup analysis of the five IFN studies, which combined outcomes of the four HCV-related and 1 HBV/HCV-related HCC studies[32], revealed that there was significantly fewer HCC recurrences in IFN groups (31.0%) than in control (63.4%) (OR: 0.26, 95% CI: 0.09-0.78, P = 0.02). There existed statistical heterogeneity (I^2 = 54%, P = 0.07).

For 3-year recurrence, the randomized model of eight studies[8,22,23,25,26,28,30,32] revealed statistical heterogeneity (I^2 = 47%, P = 0.07) and significantly fewer recurrent cases in the treatment (47.9%) than in the control (63.8%) groups (OR: 0.37, 95% CI: 0.19-0.71, P = 0.003, Figure 1C). Subgroup analysis of the five HCV-related studies[8,22,23,25,26] showed significantly fewer recurrences in IFN (52.4%) than in the control groups (69.8%) (OR: 0.41, 95% CI: 0.17-0.99, P = 0.05). Statistical heterogeneity was found among these studies (I^2 = 61%, P = 0.04). In the two HBV studies[28,30], there was a trend of fewer recurrences in the treatment groups (33.3%) than in the controls (52.9%) (OR: 0.43, 95% CI: 0.17-1.07, P = 0.07). By combining data from the five HCV-, 1[30] HBV-, and 1[32] HBV/HCV-related studies, subgroup analysis of the seven IFN studies revealed that the recurrence rate in IFN groups (48.9%) was significantly lower than in the control groups (65.2%) (OR: 0.34, 95% CI: 0.16-0.73, P = 0.006). There existed statistical heterogeneity (I^2 = 55%, P = 0.04).

For 5-year recurrence, three studies[8,22,30] showed significantly fewer HCC recurrences in IFN (66.7%) than in the control (74.3%) groups (OR: 0.32, 95% CI: 0.15-0.66, P = 0.002) with no statistical heterogeneity (I^2 = 0%, P = 0.80, Figure 1D). Subgroup analysis of the two HCV-related HCC studies[8,22] revealed significantly fewer recurrences in IFN groups (71.4%) compared with the control groups (85.1%) (OR: 0.37, 95% CI: 0.16-0.86, P = 0.02). No statistical heterogeneity was found (I^2 = 0%, P = 0.90).

Antiviral therapy and survival

For 1-year survival, combined data from six studies[8,16,23,25,28,29] showed significantly more deaths in the control (9.1%) than in the treatment (1.2%) groups (OR: 0.23, 95% CI: 0.07-0.71, P = 0.01), with no statistical heterogeneity (I^2 = 0%, P = 0.99, Figure 2A). Subgroup analysis of the three HCV studies[8,23,25] failed to show a significant difference in mortality between IFN (1.2%) and control groups (5.4%) (OR: 0.39, 95% CI: 0.07-2.27, P = 0.30). No statistical heterogeneity was found (I^2 = 0%, P = 0.95). In the two HBV studies[28,29], there was no significant difference between lamivudine (0%) and control groups (9.6%) (OR: 0.19, 95% CI: 0.02-1.55, P = 0.12) and no statistical heterogeneity (I^2 = 0%, P = 0.98). Subgroup analysis of the four IFN studies which included three HCV-related and 1 HBV/HCV-related studies[16], revealed a significantly higher mortality rate in the control (8.8%) than in IFN groups (1.7%) (OR: 0.25, 95% CI: 0.07-0.96, P = 0.04) with no statistical heterogeneity (I^2 = 0%, P = 0.91).

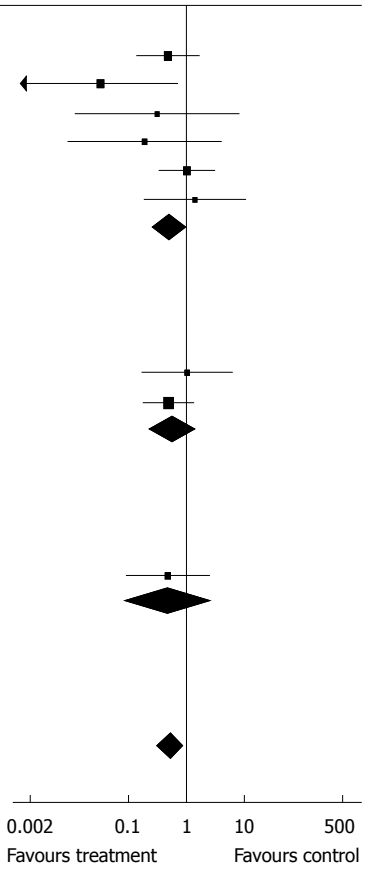
For 2-year survival, pooled data from four studies[23,25,28,29] showed a significantly lower mortality rate in the treatment groups (6.4%) compared with the control groups (22.1%) (OR: 0.31, 95% CI: 0.12-0.79, P = 0.01, Figure 2B), with no statistical heterogeneity (I^2 = 16%, P = 0.31). In subgroup analysis, the two studies of HCV-related HCC[23,25] failed to show a significant difference between IFN (6.2%) and control (18.4%) groups (OR: 0.40, 95% CI: 0.09-1.73, P = 0.22), with no statistical heterogeneity (I^2 = 0%, P = 0.54). Among the two HBV studies[28,29], the mortality in lamivudine groups (6.5%) was lower than in the control groups (24.7%) (OR: 0.27, 95% CI: 0.08-0.89, P = 0.03).

For 3-year survival, the randomized model, including eight studies[8,16,22-25,28,31], revealed statistical heterogeneity (I^2 = 46%, P = 0.07) and a significantly lower mortality rate in the treatment (12.7%) than in the control (20.8%) groups (OR: 0.43, 95% CI: 0.21-0.89, P = 0.02, Figure 2C). Subgroup analysis of the 5 HCV-related studies[8,22-25] showed significantly fewer deaths in IFN groups (7.4%) compared with the control groups (17.5%) (OR: 0.37, 95% CI: 0.15-0.88, P = 0.02), with no statistical heterogeneity (I^2 = 16%, P = 0.31). Combined outcomes of the seven IFN studies which included data from the five HCV-related and two HBV/HCV-related studies[16,31], revealed that there were significantly fewer deaths in IFN (13.4%) than in the control (19.1%) groups (OR: 0.54, 95% CI: 0.34-0.84, P = 0.006). No statistical heterogeneity was found (I^2 = 22%, P = 0.26).

For 5-year survival, four studies[8,16,22,31] showed signifi-

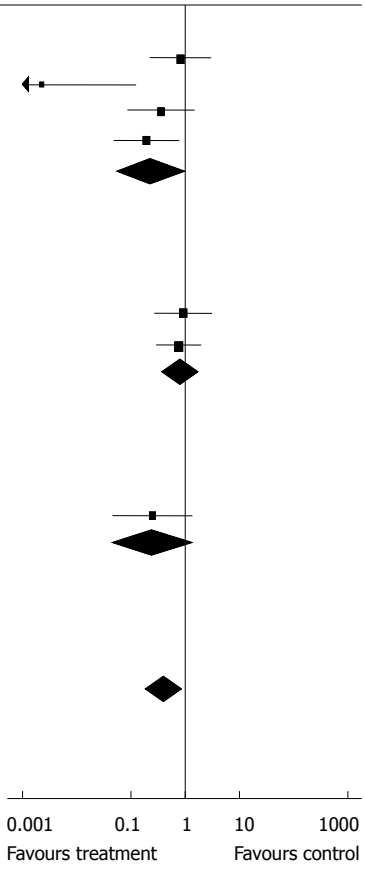
A 1-yr recurrence

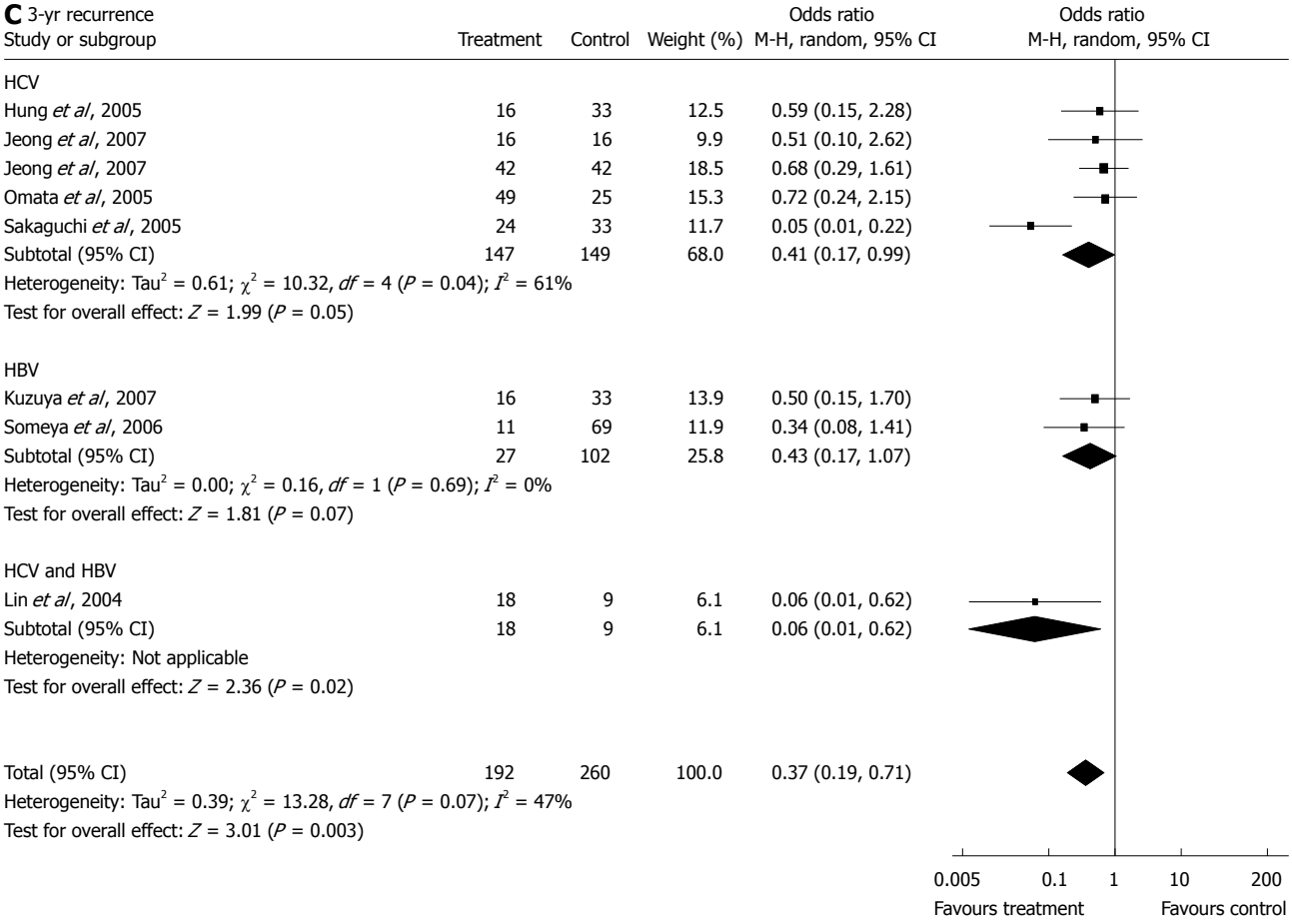
Study or subgroup	Treatment	Control	Weight (%)	Odds ratio M-H, fixed, 95% CI
HCV				
Hung *et al*, 2005	16	33	17.2	0.48 (0.14, 1.70)
Ikeda *et al*, 2000	10	10	14.9	0.03 (0.00, 0.72)
Jeong *et al*, 2007	16	16	3.5	0.31 (0.01, 8.28)
Jeong *et al*, 2007	42	42	5.9	0.19 (0.01, 4.09)
Omata *et al*, 2005	49	25	14.4	1.03 (0.33, 3.16)
Sakaguchi *et al*, 2005	24	33	3.7	1.41 (0.18, 10.78)
Subtotal (95% CI)	157	159	59.6	0.52 (0.27, 1.00)
Heterogeneity: χ^2 = 5.92, *df* = 5 (*P* = 0.31); I^2 = 16%				
Test for overall effect: *Z* = 1.95 (*P* = 0.05)				
HBV				
Kuzuya *et al*, 2007	16	33	5.5	1.04 (0.17, 6.35)
Piao *et al*, 2005	30	40	25.6	0.49 (0.18, 1.37)
Subtotal (95% CI)	46	73	31.1	0.59 (0.24, 1.43)
Heterogeneity: χ^2 = 0.49, *df* = 1 (*P* = 0.48); I^2 = 0%				
Test for overall effect: *Z* = 1.17 (*P* = 0.24)				
HCV and HBV				
Lin *et al*, 2004	18	9	9.2	0.48 (0.09, 2.56)
Subtotal (95% CI)	18	9	9.2	0.48 (0.09, 2.56)
Heterogeneity: Not applicable				
Test for overall effect: *Z* = 0.86 (*P* = 0.39)				
Total (95% CI)	221	241	100.0	0.54 (0.32, 0.89)
Heterogeneity: χ^2 = 6.41, *df* = 8 (*P* = 0.60); I^2 = 0%				
Test for overall effect: *Z* = 2.41 (*P* = 0.02)				



B 2-yr recurrence

Study or subgroup	Treatment	Control	Weight (%)	Odds ratio M-H, random, 95% CI
HCV				
Hung *et al*, 2005	16	33	16.3	0.82 (0.22, 3.04)
Ikeda *et al*, 2000	10	10	3.3	0.00 (0.00, 0.13)
Jeong *et al*, 2007	16	16	14.9	0.36 (0.09, 1.51)
Sakaguchi *et al*, 2005	24	33	15.3	0.19 (0.05, 0.78)
Subtotal (95% CI)	66	92	49.8	0.23 (0.06, 0.99)
Heterogeneity: Tau^2 = 1.30; χ^2 = 8.70, *df* = 3 (*P* = 0.03); I^2 = 65%				
Test for overall effect: *Z* = 1.97 (*P* = 0.05)				
HBV				
Kuzuya *et al*, 2007	16	33	17.2	0.92 (0.27, 3.16)
Piao *et al*, 2005	30	40	20.7	0.76 (0.29, 1.98)
Subtotal (95% CI)	46	73	37.8	0.82 (0.38, 1.74)
Heterogeneity: Tau^2 = 0.00; χ^2 = 0.06, *df* = 1 (*P* = 0.81); I^2 = 0%				
Test for overall effect: *Z* = 0.52 (*P* = 0.60)				
HCV and HBV				
Lin *et al*, 2004	18	9	12.4	0.25 (0.05, 1.36)
Subtotal (95% CI)	18	9	12.4	0.25 (0.05, 1.36)
Heterogeneity: Not applicable				
Test for overall effect: *Z* = 1.60 (*P* = 0.11)				
Total (95% CI)	130	174	100.0	0.42 (0.19, 0.90)
Heterogeneity: Tau^2 = 0.52; χ^2 = 12.16, *df* = 6 (*P* = 0.06); I^2 = 51%				
Test for overall effect: *Z* = 2.22 (*P* = 0.03)				





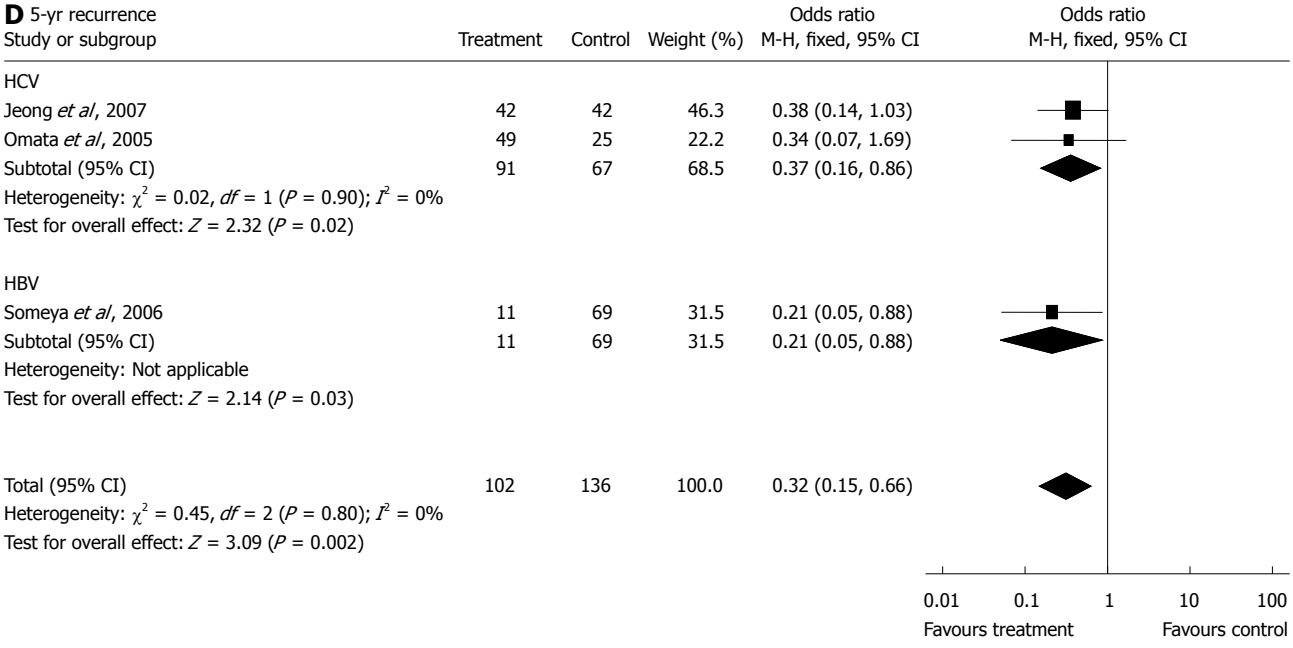
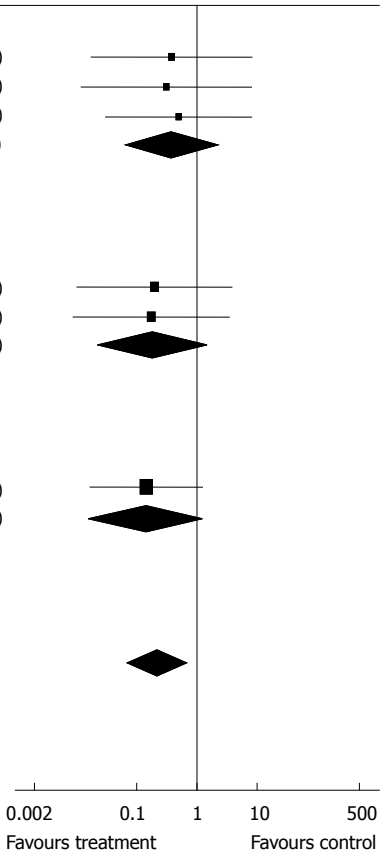


Figure 1 Forest plot of randomized (RCTs) and non-randomized (NRCTs) controlled trials evaluating the effect of post-operative antiviral therapy on hepatocellular carcinoma (HCC) recurrence after curative therapy (RevMan, version 5; The Cochrane Collaboration, Copenhagen, Denmark). A: 1-year recurrence, the Mantel-Haenszel method; B: 2-year recurrence, the Der Simonian and Laird method; C: 3-year recurrence, the Der Simonian and Laird method; D: 5-year recurrence, the Mantel-Haenszel method.

cantly fewer deaths in IFN (25.1%) than in the control (42.0%) groups (OR: 0.42, 95% CI: 0.27-0.66, $P = 0.0002$), with no statistical heterogeneity ($I^2 = 42\%$, $P = 0.16$, Figure 2D). Subgroup analysis of the two HCV-related studies[8,22], using the randomized model, failed to reveal a significant difference between IFN (71.4%) and control

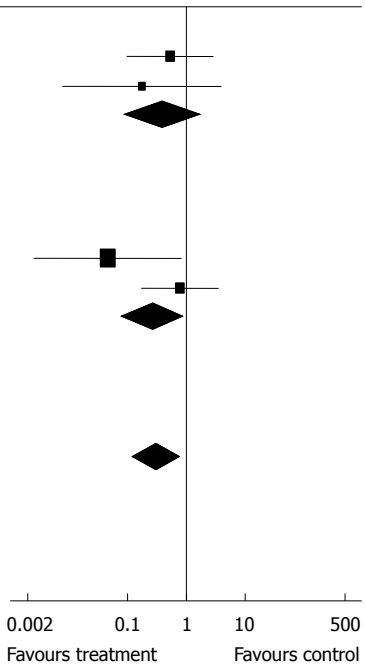
A 1-yr mortality

Study or subgroup	Treatment	Control	Weight (%)	Odds ratio M-H, fixed, 95% CI
HCV				
Hung *et al*, 2005	16	33	10.0	0.38 (0.02, 8.43)
Jeong *et al*, 2007	16	16	9.0	0.31 (0.01, 8.28)
Omata *et al*, 2005	49	25	8.1	0.50 (0.03, 8.34)
Subtotal (95% CI)	81	74	27.2	0.39 (0.07, 2.27)
Heterogeneity: χ^2 = 0.05, *df* = 2 (*P* = 0.98); I^2 = 0%				
Test for overall effect: *Z* = 1.04 (*P* = 0.30)				
HBV				
Kuzuya *et al*, 2007	16	33	18.1	0.20 (0.01, 3.92)
Piao *et al*, 2005	30	40	18.4	0.18 (0.01, 3.53)
Subtotal (95% CI)	46	73	36.5	0.19 (0.02, 1.55)
Heterogeneity: χ^2 = 0.00, *df* = 1 (*P* = 0.95); I^2 = 0%				
Test for overall effect: *Z* = 1.55 (*P* = 0.12)				
HCV and HBV				
Lo *et al*, 2007	40	40	36.3	0.15 (0.02, 1.27)
Subtotal (95% CI)	40	40	36.3	0.15 (0.02, 1.27)
Heterogeneity: Not applicable				
Test for overall effect: *Z* = 1.75 (*P* = 0.08)				
Total (95% CI)	167	187	100.0	0.23 (0.07, 0.71)
Heterogeneity: χ^2 = 0.64, *df* = 5 (*P* = 0.99); I^2 = 0%				
Test for overall effect: *Z* = 2.57 (*P* = 0.01)				



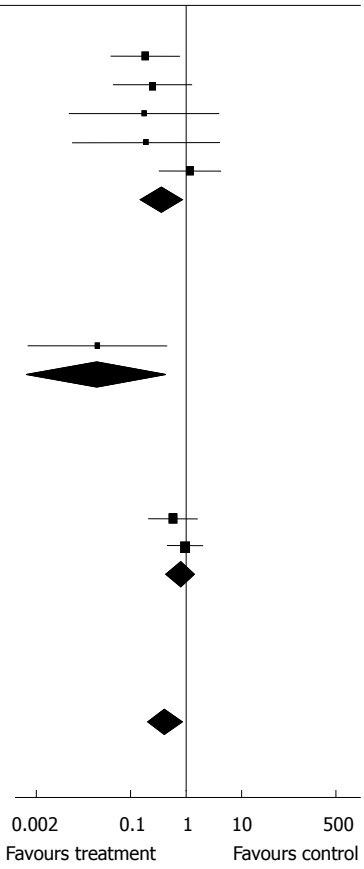
B 2-yr mortality

Study or subgroup	Treatment	Control	Weight (%)	Odds ratio M-H, fixed, 95% CI
HCV				
Hung *et al*, 2005	16	33	21.0	0.53 (0.10, 2.91)
Jeong *et al*, 2007	16	16	12.8	0.18 (0.01, 3.97)
Subtotal (95% CI)	32	49	33.8	0.40 (0.09, 1.73)
Heterogeneity: χ^2 = 0.37, *df* = 1 (*P* = 0.54); I^2 = 0%				
Test for overall effect: *Z* = 1.23 (*P* = 0.22)				
HBV				
Kuzuya *et al*, 2007	16	33	45.9	0.05 (0.00, 0.83)
Piao *et al*, 2005	30	40	20.3	0.78 (0.17, 3.54)
Subtotal (95% CI)	46	73	66.2	0.27 (0.08, 0.89)
Heterogeneity: χ^2 = 3.30, *df* = 1 (*P* = 0.07); I^2 = 70%				
Test for overall effect: *Z* = 2.15 (*P* = 0.03)				
Total (95% CI)	78	122	100.0	0.31 (0.12, 0.79)
Heterogeneity: χ^2 = 3.57, *df* = 3 (*P* = 0.31); I^2 = 16%				
Test for overall effect: *Z* = 2.47 (*P* = 0.01)				



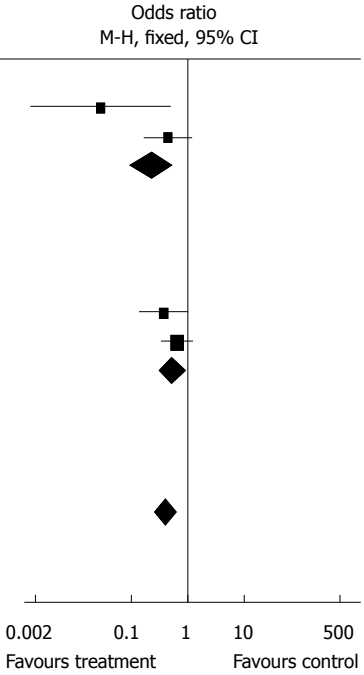
C 3-yr mortality

Study or subgroup	Treatment	Control	Weight (%)	Odds ratio M-H, random, 95% CI
HCV				
Akamatsu *et al*, 2006	53	399	14.2	0.18 (0.04, 0.77)
Hung *et al*, 2005	16	33	12.2	0.25 (0.05, 1.29)
Jeong *et al*, 2007	16	16	4.7	0.18 (0.01, 3.97)
Jeong *et al*, 2007	42	42	4.8	0.19 (0.01, 4.09)
Omata *et al*, 2005	49	25	15.9	1.18 (0.32, 4.29)
Subtotal (95% CI)	176	515	51.7	0.37 (0.15, 0.88)
Heterogeneity: Tau^2 = 0.16; χ^2 = 4.75, *df* = 4 (*P* = 0.31); I^2 = 16%				
Test for overall effect: *Z* = 2.24 (*P* = 0.02)				
HBV				
Kuzuya *et al*, 2007	16	33	5.3	0.03 (0.00, 0.46)
Subtotal (95% CI)	16	33	5.3	0.03 (0.00, 0.46)
Heterogeneity: Not applicable				
Test for overall effect: *Z* = 2.49 (*P* = 0.01)				
HCV and HBV				
Lo *et al*, 2007	40	40	19.4	0.58 (0.21, 1.63)
Mazzaferro *et al*, 2006	76	74	23.6	0.97 (0.46, 2.04)
Subtotal (95% CI)	116	114	42.9	0.81 (0.44, 1.49)
Heterogeneity: Tau^2 = 0.00; χ^2 = 0.60, *df* = 1 (*P* = 0.44); I^2 = 0%				
Test for overall effect: *Z* = 0.68 (*P* = 0.50)				
Total (95% CI)	308	662	100.0	0.43 (0.21, 0.89)
Heterogeneity: Tau^2 = 0.45; χ^2 = 12.89, *df* = 7 (*P* = 0.07); I^2 = 46%				
Test for overall effect: *Z* = 2.28 (*P* = 0.02)				



D 5-yr mortality

Study or subgroup	Treatment	Control	Weight (%)	Odds ratio M-H, fixed, 95% CI
HCV				
Jeong *et al*, 2007	42	42	20.9	0.03 (0.00, 0.50)
Omata *et al*, 2005	49	25	19.6	0.45 (0.17, 1.20)
Subtotal (95% CI)	91	67	40.5	0.23 (0.10, 0.54)
Heterogeneity: χ^2 = 3.76, *df* = 1 (*P* = 0.05); I^2 = 73%				
Test for overall effect: *Z* = 3.38 (*P* = 0.0007)				
HCV and HBV				
Lo *et al*, 2007	40	40	21.6	0.38 (0.14, 1.02)
Mazzaferro *et al*, 2006	76	74	37.9	0.65 (0.34, 1.25)
Subtotal (95% CI)	116	114	59.5	0.55 (0.32, 0.95)
Heterogeneity: χ^2 = 0.82, *df* = 1 (*P* = 0.37); I^2 = 0%				
Test for overall effect: *Z* = 2.16 (*P* = 0.03)				
Total (95% CI)	207	181	100.0	0.42 (0.27, 0.66)
Heterogeneity: χ^2 = 5.14, *df* = 3 (*P* = 0.16); I^2 = 42%				
Test for overall effect: *Z* = 3.76 (*P* = 0.0002)				



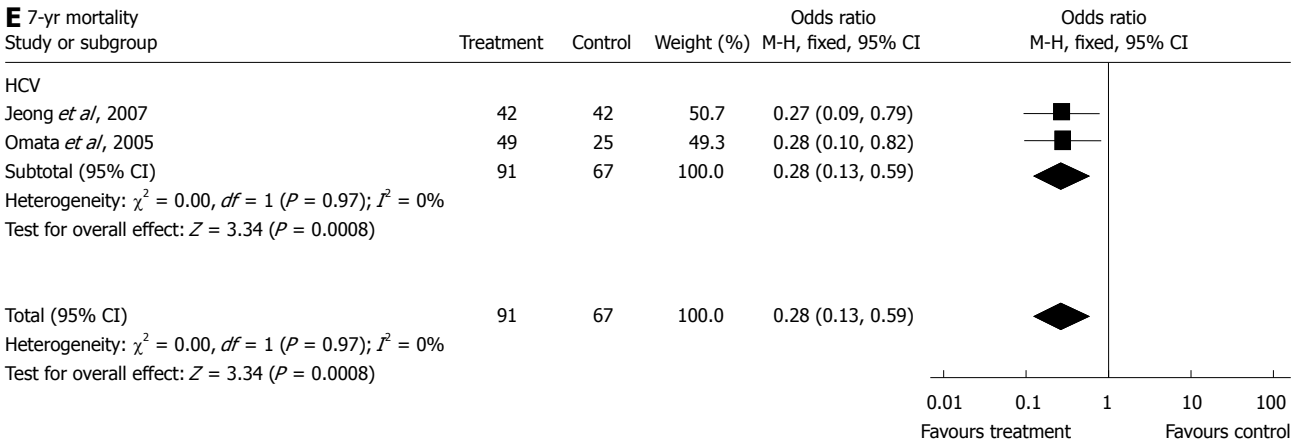


Figure 2 Forest plot of randomized (RCTs) and non-randomized (NRCTs) controlled trials evaluating the effect of post-operative antiviral therapy on HCC mortality after curative therapy (RevMan, version 5; The Cochrane Collaboration, Copenhagen, Denmark). A: 1-year mortality, the Mantel-Haenszel method. B: 2-year mortality, the Mantel-Haenszel method; C: 3-year mortality, the Der Simonian and Laird method; D: 5-year mortality, the Mantel-Haenszel method; E: 7-year mortality, the Mantel-Haenszel method.

(85.1%) (OR: 0.15, 95% CI: 0.01-2.67, $P = 0.20$) groups with a statistical heterogeneity ($I^2 = 73\%$, $P = 0.05$).

For 7-year survival, combined data from two HCV-related studies[8,22] including 158 patients revealed a significantly lower mortality rate in IFN (31.9%) than in the control (52.2%) groups (OR: 0.28, 95% CI: 0.13-0.59, $P = 0.0008$), with no statistical heterogeneity ($I^2 = 0\%$, $P = 0.97$, Figure 2E).

DISCUSSION

The prevention of recurrence constitutes one of the most important challenges to improve the prognosis of patients with HCC after curative therapies. Currently, there is insufficient evidence to reach a consensus on appropriate adjuvant therapies for secondary prevention. In the present meta-analysis, postoperative antiviral therapy as a whole has been shown to reduce HCC recurrence at year 1, 2, 3, and 5. Subgroup analyses have further demonstrated that this effect of reduction remained significant in IFN-treated HCCs as well as pure HCV-associated HCCs.

Generally, recurrences can be differentiated into early or late ones. Early HCC recurrences were assumed to be derived from residues of original HCC after a potentially curative therapy and usually appear within the first 2 years. Late recurrences, which typically occur more than 2 years after the elimination of primary tumors, develop on the basis of underlying liver diseases, resulting from new carcinogenesis[14]. Persistent active hepatitis is common in advanced stages of chronic HBV or HCV infection, including HCC. The prevalence of multicentric carcinogenesis increases during progression to active hepatitis and hepatic fibrosis[33]. Hepatocyte necrosis and inflammation may be closely involved in multifocal recurrence in chronic HBV/HCV-infected patients who had complete resection or ablation of HCC tumors. Hence, it is hypothesized that antiviral therapies would help prevent HCC recurrence by cleaning the carcinogenic soil and eliminating possibilities of novel tumorigenesis through their viral suppression and anti-inflammation action.

There have been studies indicating that IFN therapy for chronic hepatitis C has improved the histological features of hepatic inflammation and fibrosis, induced remission of active hepatitis, and thereby hindered HCC development[34,35]. Similarly, IFN may also suppress multifocal recurrence arising from multicentric carcinogenesis after resection or ablation in the same way. Nonetheless, the results of this meta-analysis have shown that IFN not only reduced late recurrent HCCs but also early ones, especially in patients with pure HCV infection, which indicates that more than one mechanism may actually contribute to the benefit of adjuvant therapy with IFN in lowering recurrent HCCs. Beside immunostimulant, antiviral, antiangiogenic, and anticarcinogenic properties, IFN has been found to have *in vitro* and *in vivo* antiproliferative and tumoricidal effects against HCC as well[1,16,36]. Therefore, postoperative IFN therapy might be of advantage to recurrence prevention due to a combination of chemopreventive effects on multicentric carcinogenesis and antiproliferative and tumoricidal effects on residual HCC cells.

Compared with recurrence, although the overall results of antiviral therapies or IFN alone at various years were almost positive in improving survival, subgroup analyses of HCV-related HCCs produced mixed results. For survival at year 1, 2, and 5, current data are insufficient to give out significant results; for survival at year 7, given the condition that only two studies with a total of 158 patients were available for analysis, the significant difference between IFN and control groups should be taken with a grain of salt; only the benefit for 3-year survival was based on a relatively larger sample size, but it also needs further confirmation by even larger studies.

Sustained virological response (SVR) is defined on the basis of the absence of serum HCV RNA at least 6 mo after completion of IFN therapy. All seven studies of pure HCV-related HCCs presented information on the response to IFN therapy (Table 1); however, the effects varied greatly as different IFN formulas with different doses, intervals, and durations were applied in different

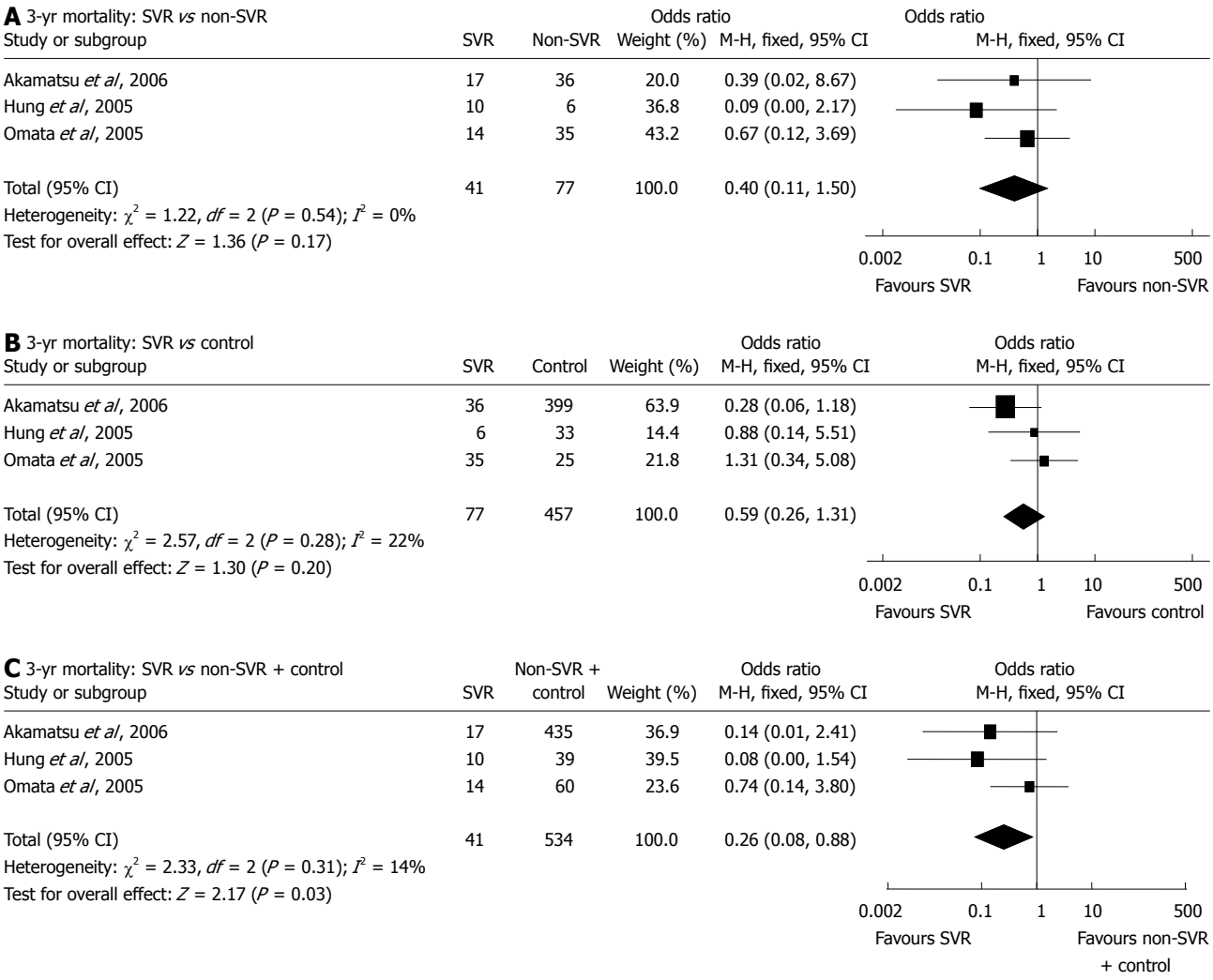


Figure 3 Forest plot of randomized (RCTs) and non-randomized (NRCTs) controlled trials evaluating the effect of post-operative antiviral therapy on the 3-year mortality of HCV-related HCC patients after curative therapy (RevMan, version 5; The Cochrane Collaboration, Copenhagen, Denmark). A: Comparison between patients with sustained virologic response (SVR) and those without sustained virologic response (non-SVR), the Mantel-Haenszel method; B: Comparison between non-SVR patients and control subjects, the Mantel-Haenszel method; C: Comparison between SVR patients and non-SVR patients plus control subjects, the Mantel-Haenszel method.

patient populations. The comparisons between patients with SVR and those without SVR or IFN treatment were performed in four studies; all showed a certain superiority of SVR, either in survival or the first/second HCC recurrence[8,22,24,25]. As among all nine endpoints, i.e. 1, 2, 3 and 5-year recurrence and 1, 2, 3, 5 and 7-year survival, only the data on the 3-year survival of SVR and non-SVR patients were available in two or more studies, a subgroup analysis was able to be performed. Although pooled data from three studies[8,24,25] did not reveal significant difference in the 3-year mortality between SVR patients (2.4%) and non-SVR ones (14.3%) (OR: 0.40, 95% CI: 0.11-1.50, $P = 0.17$, Figure 3A), or between non-SVR patients and control subjects (18.8%) (OR: 0.59, 95% CI: 0.26-1.31, $P = 0.20$, Figure 3B), it was shown that the mortality rate in SVR patients was significantly lower than that in non-SVR patients plus control ones (18.2%) (OR: 0.26, 95% CI: 0.08-0.88, $P = 0.03$, Figure 3C). Therefore, for HCV-related HCC, it may be worth considering whether it is reasonable to take SVR as a target of antiviral therapies in order to improve prognosis. If so, intensified or combined treatment, such as the combination of PEG-IFN and ribavirin, would be indicated as long as the adverse effects are well balanced and patients are tolerant to such treatment.

Regarding HBV-related HCC, analysis on the two lamivudine studies that provided data on recurrence and mortality at year 1, 2, and 3 failed to demonstrate significant differences but only a trend of superiority in late recurrence, although it showed significant benefit in the 2- and 3-year survival. Besides problems with the limited number of studies and small sample sizes, it may be partially explained by the fact that the emergence of YMDD mutations (e.g. rtM204V/I) reduces the benefit of lamivudine therapy, which is relatively frequent in clinical trials and generally associated with worse clinical outcomes, such as hepatitis flares, hepatic decompensation, fatal liver failure and death[28,37]. *In vitro* studies have shown that the rtM204V/I mutations decreased susceptibility of the viral polymerase to lamivudine and reduced functional capacity

of the viral polymerase[37]. As a result, serum HBV DNA remains at or relapses to a high level, which leaves the liver prone to HCC recurrence, as shown by one report that, during a median follow-up of 35 mo, patients in the non-viraemia (< 100 000 copies/mL) group had a lower 5-year cumulative recurrence rate than those in the viraemia group (54.7% *vs* 72.9%, $P = 0.043$), and sustained viraemia independently increased recurrence after surgery in multivariate analysis ($P = 0.041$)[38]. Therefore, antiviral therapy may be indicated in patients with detectable serum HBV DNA since lower viral load is important to reduce HCC recurrence; meanwhile, periodic assessment of serum HBV DNA using a sensitive assay should be applied to monitor the antiviral resistance. It is hypothesized that potent oral NAs with higher genetic barrier to resistance, such as entecavir, could overcome this disadvantage, but it needs further studies to confirm.

In conclusion, this meta-analysis demonstrates the benefit of antiviral treatment, IFN in particular, in reducing short-term and long-term HCC recurrence and mortality after curative therapies such as hepatic resection or ablation for primary HCC. The effects seem particularly striking for patients with pure HCV infection, and patients who have achieved SVR after IFN therapy may expect fewer recurrences and longer survival. These data are highly encouraging and should call for further evaluation and development of new regimens. For HBV-related HCCs, future randomized control trials with larger sample size, longer follow-up, and regular HBV DNA monitoring will be needed to substantiate the beneficial effects of antiviral therapies, such as IFN and entecavir, on the prognosis of HCC. Finally, based on these results, we hypothesize that if compared with other adjuvant therapies, such as systematic chemotherapy, molecular targeted therapy, some locoregional therapies, antiviral therapy may serve as a cost-effective and favorable alternative to improve the prognosis of patients with HBV/HCV-related HCCs because the underlying carcinogenic background can be reformed, which also warrants in-depth investigations.

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COMMENTS

Background

Hepatocellular carcinoma (HCC) is common worldwide, particularly in Asia where chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infection is the most common etiology. The prevention of recurrence remains as one of the most important challenges to improve the prognosis after curative therapies. Up till now, although several clinical trials have evaluated adjuvant antiviral therapies with interferon or lamivudine, no clear recommendations have been proposed.

Research frontiers

As a quantitative technique for therapeutic evaluation, meta-analysis may be used when controversy exists, particularly when trials have insufficient statistical power. In the present study, this mathematical tool is utilized to verify the hypothesis that antiviral therapy may serve as an alternative strategy to improve the prognosis of patients with HBV/HCV-related HCCs.

Innovations and breakthroughs

Although antiviral therapies using interferons or lamivudine have shown promising results in chronic hepatitis B or hepatitis C, their effects on patients with HCC after curative therapy remain controversial, mainly due to the small sample size and short follow-up time. By pooling data from available literature, the present study for the first time demonstrates the benefit of antiviral therapies, interferon in particular, in reducing both short-term and long-term HCC recurrence as well as mortality after curative therapies. The effects seem more remarkable for patients with HCV-related HCC. For HBV-related HCCs, this study warrants future randomized control trials with larger sample size and longer follow-up. This study intends to remind surgeons that basic treatment that targets underlying carcinogenic background might be more efficient than the so-called novel techniques which are attractive but still questionable.

Applications

The results of study indicate that postoperative antiviral therapy, interferon in particular, may serve as a favorable alternative to reduce recurrence and mortality in patients with HBV/HCV-related HCCs.

Peer review

This is a meta-analysis of postoperative antiviral treatment on tumor recurrence and survival in patients with chronic HBV or HCV infection-related HCC after curative therapy, which has been an interesting issue but still has no solid evidence. The authors conclude that postoperative antiviral treatment may reduce short-term and long-term HCC recurrence and mortality after curative therapy. Antiviral therapy may serve as a favorable alternative to improve the prognosis of patients with HBV/HCV-related HCCs. The authors have nicely analyzed the recent publications. Overall, these results are well laid out and logical.

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BRIEF ARTICLE

Retrospective analysis of perianal Paget's disease with underlying anorectal carcinoma

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Abstract

AIM: To analyze clinical and pathological characteristics of an aggressive subtype of perianal Paget's disease (PPD) and explore its rational treatment modalities.

METHODS: PPD patients were retrospectively collected in the institutional colorectal database of the Fudan University Shanghai Cancer Center. Detailed patient histories of past medical condition, diagnosis, treatment, and pathological findings were reviewed. Surgical specimen from diagnosis and surgery were reviewed by two independent pathologists for confirmation of diagnoses. Follow up was accomplished by clinical interview by cellphone.

RESULTS: In total, eight cases of PPD were analyzed. All patients had underlying anorectal adenocarcinoma, including seven with synchronous lesions and one with metachronous lesions. Moreover, all anorectal lesions had a mucin-producing component. The median age at diagnosis was 65 (range 29-81 years), and the male/female ratio was 7:1. The Median follow-up time of all patients was 61.5 mo (range 10-204 mo). One patient treated with abdominoperineal resection (APR) died from lung metastases 10 mo after the APR operation. The other patients are still free of disease at the time of this analysis.

CONCLUSION: PPD is a rare malignancy and is easily misdiagnosed. Underlying anorectal cancer was not unusual and was a significant prognostic factor. Rational treatment of both anorectal cancer and PPD lesion is essential for long-term survival.

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Key words: Perianal Paget's disease; Anorectal cancer; Treatment; Prognosis

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INTRODUCTION

Perianal Paget's disease (PPD) is a rare malignancy and less than 200 cases have been reported in literature. The prognosis of simple PPD is relatively favorable, both overall and disease-free survival are approximately 60% at 5-year[1-3].

However, one subtype of this malignancy, PPD with synchronous or metachronous carcinoma of the anorectum, has a poor prognosis, based on limited reports on this entity[1-5]. Due to the rarity of this condition, most of the published literature presented limited numbers of case reports. The natural course of the disease and its optional management is still unknown.

The current report aims to increase the current knowledge of PPD with underlying adenocarcinoma of the anorectum by retrospectively reviewing the natural history and treatment outcome of eight consecutive and non-selected patients diagnosed with this rare condition.

MATERIALS AND METHODS

All cases of PPD diagnosed and treated between January 1985 and December 2009 at the colorectal surgical service of Fudan University Shanghai Cancer Center were reviewed retrospectively. According to the institutional colorectal database, patients with histological confirmation of Paget's disease originating from the perianal area, as well as adenocarcinoma of the rectum and/or anal canal, were included in this analysis.

Detailed patient histories of past medical conditions, diagnosis, treatment, and pathological findings were reviewed.

Preoperative workup included fiberoptic full colonoscopy, computed tomography and/or magnetic resonance imaging of the abdomen and pelvis, and carcinoembryonic antigen tumor marker analysis to confirm the lesions of the perianal area and anorectum and to exclude lesions in other part of the colon.

Surgical specimens from diagnosis and surgery were reviewed by two independent pathologists for confirmation of diagnoses of both Paget's disease of the perianal area and anorectal adenocarcinoma. Only when the preoperative colonoscopy and postoperative pathological examination confirmed that there was no continuity between the two lesions through mucosa spread and interstitial invasion, were the patients included in the study.

Follow up was accomplished according to the NCCN guideline V2.2009 for rectal cancer and survival information was achieved by clinical and/or cellphone interview.

RESULTS

Patients' characteristics and diagnosis

Eight cases were diagnosed with PPD with synchronous or metachronous anorectal adenocarcinoma. Patients' characteristics are detailed in Table 1. The median age at diagnosis was 65 years (range 29-81 years), and the male/female ratio was 7:1. The median follow-up time of all patients was 61.5 mo (range 10-204 mo). One patient treated with APR died from lung metastases 10 mo after the APR operation. The other seven patients are still free of disease at the time of this analysis, one of whom had local recurrence of PPD and received salvage wide local excision.

Eczema-like symptoms including eczema-like skin change, itching, and pain were the most common presenting symptoms and occurred in five of eight cases as the only initial finding. Digestive tract symptoms, including hematochezia, increased frequency of defecation without diarrhea, and constipation, respectively, were observed in 3, 2, and 1 cases of the remaining three cases. The median time from symptom onset to diagnosis of Paget's disease was 27.3 mo (range 6-96 mo).

Table 1 General patient information

Patient	Gender	Age (yr)	Disease status	Treatment
1	Male	80	Synchronous	APR
2	Male	29	Synchronous	APR
3	Female	68	Synchronous	APR
4	Male	63	Synchronous	APR
5	Male	53	Synchronous	Excision biopsy of anorectal lesion + radical radiation therapy for both Paget's disease and anorectal cancer
6	Male	61	Synchronous	APR
7	Male	81	Synchronous	APR
8	Male	70	Metachronous	Radiation therapy; Inguinal node resection and APR for recurrence

APR: Abdominoperineal resection.

Six patients were pathologically diagnosed as PPD before surgery or definitive radiation, and pathological confirmation of PPD was obtained from specimens after radical surgery in two additional cases.

Treatment and clinical outcome

All patients received treatment for curative intent including surgery and radiation therapy. Seven patients received radical surgery. Five of the seven patients were treated with abdominoperineal resection (APR), and one had trans-anal excision followed by APR.

One patient was treated with induction radiation therapy to 50.4 Gy delivered over 5.5 wk (1.8 Gy/daily fraction) as the initial treatment for Paget's disease. He developed local recurrence of Paget's disease and lymphadenopathy in the left inguinal area one year after the completion of radiation therapy, and was diagnosed with adenocarcinoma of the lower rectum. The patient was treated with APR and adjuvant chemotherapy. One additional patient received excision biopsy of the rectal cancer followed by definitive radical radiation therapy then adjuvant chemotherapy. Radical surgery was not performed for rectal cancer due to his poor general health. The total dose for definitive radiation therapy was 59.4 Gy given at 1.8 Gy/daily fractions. Adjuvant chemotherapy (five cycles of capecitabine) was used in two patients.

Pathology study

Pathological study of the resected specimens of seven surgically treated patients and excisional biopsy of one case treated with definitive radiation revealed typical characteristics of Paget's cells as depicted by Marchesa *et al*[1]: large cytologically malignant cells (Paget cells) that had vesicular nuclei and prominent nucleoli and contained abundant pale or granular, sometimes vacuolated, cytoplasm.

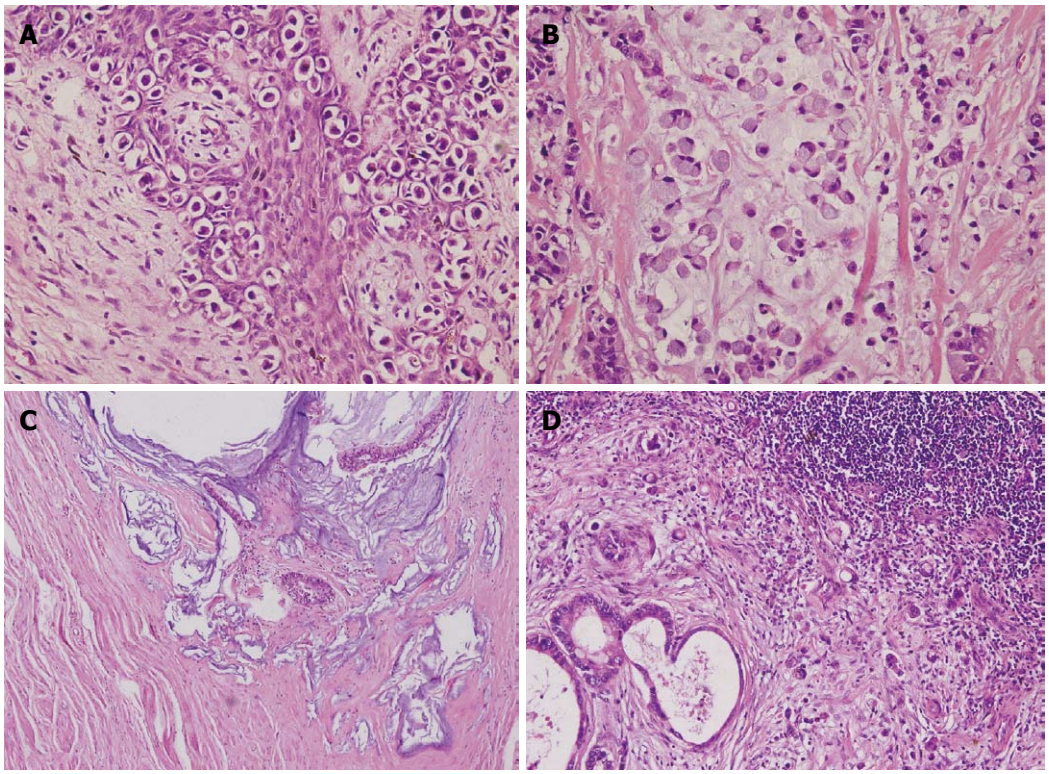


Figure 1 Histopathological images of perianal Paget's disease, rectal cancer, and metastases in a lymph node from one patient. A: Paget's disease under high-power magnification (× 400); B: Signet cell ring cancer component of rectal cancer under high-power magnification (× 400); C: Mucinous adenocarcinoma component of rectal cancer under high-power magnification (× 200); D: Metastases in inguinal lymph node under high-power magnification (× 400).

Table 2 Clinicopathological factors (mean ± SE) *n* (%)

Factors	Value
Distance to anal edge of anorectal cancer (cm)	2.69 ± 0.70
Diameter of anorectal cancer (cm)	3.13 ± 1.76
Diameter of PPD (cm)	4.81 ± 2.59
Dental line involving (%)	100
Mucin producing component	8/8 (100)
Mucinous adenocarcinoma	5/8 (62.5)
Signet ring cell cancer	1/8 (12.5)
Mix typed	2/8 (25)
Stages of anorectal cancer	
Ⅰ	5/8 (62.5)
Ⅱ	2/8 (25)
Ⅲ	1/8 (12.5)

PPD: Perianal Paget's disease.

Moreover, all cases had Paget's disease confined in the perianal area with clear margins from their synchronous anorectal adenocarcinoma. For the only case treated with definitive radiation therapy, colonoscopy before surgery showed a clear distance, without evidence of disease, between Paget's disease and the lower rectal lesion, and a clear margin was observed from the excised specimen from the rectum. Of all cases, the underlying adenocarcinoma originated from the lower rectum and involved the dentate line. All anorectal lesions had a mucin-producing component, which had rarely been reported previously in the published literature. Clinicopathological features of the seven surgically treated cases are detailed in Table 2. Figure 1 illustrates histopathological images of PPD, anorectal adenocarcinoma, and metastases in a lymph node from one typical case.

DISCUSSION

Paget's disease is an intra-epithelial adenocarcinoma and was first reported in the breast by Sir Paget J in 1874. Since then, Paget's cells and Paget's disease have been reported in several extramammary sites, including the axilla, perineum, groin, thigh, scrotum, and vulva[6]. In 1893, Darrier and Coulillaud reported the first case of Paget's disease in the perianal region.

PPD is often referred to as an intraepithelial adenocarcinoma within 6 cm scope around the anus and below the dentate line. Signet ring cells with pale, vacuolated cytoplasm are present, which are usually positive with a periodic acid-Schiff mucin stain under microscopic examination[7,8].

There is considerable controversy about the origin of PPD. Helwig *et al*[9] considered perianal and vulval Paget's disease to be a manifestation of a multicentric effect of an unknown carcinogenic stimulus on apocrine structures, epidermis, and glandular elements of the rectum and the urethra. Otherwise, some researchers observed histochemical similarity between the mucin in the intraepidermal cells and the underlying carcinoma cells, and formed the view that the cells in the epidermis represented metastatic spread[10-14]. In our group, eight anorectal carcinomas also had mucin-producing components, such as mucinous adenocarcinoma, signet ring cell cancer, or

mixed type. However, fiberoptic full colonoscopy confirmed disease-free distance between Paget's disease and the anorectal carcinoma. Moreover, pathologists failed to find continuity between the two lesions. This result reinforced the supposition of "metastatic origination of perianal Paget's disease" from a much bigger sample size basis. The mucin secretion character of the anorectal lesion and clinical manifestation of mucinous discharges suggested that mucin might play an important role in the histogenesis of PPD. We hypothesized that mucin may function in Paget's disease formation by two ways: cancer cell produced-mucin might stimulate carcinogenesis of apocrine structures/epidermis in the perianal area directly. On the other hand, mucinous tumor cells might infiltrate through the epidermal barrier and form a cancer nest beneath.

After PPD was first reported by Darrier and Coulilaud in 1893, new cases emerged gradually[4,15-19], but systemic analysis of this disease was scarce. Only in prestigious medical institutes, such as the Memorial Sloan-Kettering Cancer Center, the Mayo Clinic, and the Cleveland Clinic Foundation, was this type of disease collected and analyzed systemically; however, the sample sizes were very small (27, 13, and 14 cases, respectively)[1-3]. Unfortunately, these studies focused on simple PPD, and the aggressive subtype with underlying anorectal carcinoma was still unknown.

Interestingly, all patients with PPD in our group had underlying adenocarcinoma in the anorectum. Therefore, we attempted to summarize the natural history, clinicpathological characteristics, treatment experiences, and information about survival to boost the knowledge of this rare condition.

Eczema-like symptoms were the most common initial complaints, including eczema-like skin change, itching, and pain, which were easily underestimated by the patients themselves and by physicians. On the other hand, digestive symptoms could be the first presentation, but are usually ignored by the patients themselves. In our group, the median time from symptom onset to diagnosis of Paget's disease was 27.3 mo. PPD with underlying anorectal carcinoma in has been rarely reported. In some cases, anorectal carcinoma was neglected in the diagnosis and treatment modality after confirmation the diagnosis of Paget's disease. A total of 155 cases of Paget's disease have been reported. The median age of onset was 65.3 years and male/female ratio was 1.06. The proportion of underlying anorectal cancer was as high as 41.8%.

A wide range of treatment modalities have been previously reported, including surgery (wide local excision with or without reconstruction and grafting[20-23], abdominoperineal resection[1-3], Mohs micrographic surgery[24]), radiotherapy[25], chemo-radiotherapy[26-28], and photodynamic therapy[29,30].

Surgical methods are still the mainstay in the management of PPD. Table 3 shows the stage and management recommended by Shutze *et al*[31] in 1990 and followed by most oncologists.

According to this staging system, seven PPD with synchronous anorectal carcinoma should be grouped as stage Ⅱ B and APR should be recommended. For patient 8 in Table 1, he was diagnosed as stage Ⅲ metachronous anorectal cancer and radiation therapy, inguinal lymph node dissection, and APR were carried out sequentially.

Table 3 Stage and management of PPD

Stage	Description	Management
Ⅰ	Paget's cells found in perianal epidermis and adnexae without primary carcinoma	WLE
Ⅱ A	Cutaneous Paget's disease with associated adnexal carcinoma	WLE
Ⅱ B	Cutaneous Paget's disease with associated anorectal carcinoma	APR
Ⅲ	Paget's disease in which associated carcinoma has spread to regional nodes	ILND + WLE/APR
Ⅳ	Paget's disease with distant metastases of associated carcinoma	CT + RT + LPM

WLE: Wide local excision; ILND: Inguinal lymph node dissection; CT: Chemotherapy; RT: Radiotherapy; LPM: Local palliative management.

Brown *et al*[25] reported six cases of PPD treated by radiotherapy as adjuvant or radical modality. They found that PPD had a high recurrence rate after radiotherapy. However, only three patients had an associated underlying anal carcinoma: one 9 years pre-diagnosis of PPD, one at diagnosis (both adeno-and squamous carcinoma), and one 10 years after diagnosis of PPD. Reports of chemoradiation are also infrequent. Although promising, there have not been many subsequent reports following these initial cases[26-28]. In our study, two patients received radical radiotherapy and adjuvant chemotherapy with capecitabine. One patient had recurrence and inguinal lymph metastasis, and an APR operation confirmed the existence of underlying anorectal cancer. Subsequently, he had local recurrence in the perineum and received wide local excision and is presently tumor-free. The other received transanal excision of an anorectal lesion and followed by radical radiation therapy for both Paget's disease and rectal cancer, and five cycles of adjuvant chemotherapy with capecitabine. No evidence of recurrence has been found at the latest follow. The results of this research are promising; however, further research is required.

Photodynamic therapy has also been successfully used in early perianal skin tumors, such as Bowen's disease and PPD[29,30]. However, because of limitation of conditions, this technique could not be used in our patients.

Survival of simple PPD was favorable. McCarter *et al*[3] collected 27 cases of PPD in the Memorial Sloan-Kettering Cancer Center, and most patients (74%) were treated with wide excision. At a median follow-up of 67 mo, 56 percent (15/27) had no evidence of disease, and two patients had died of metastatic disease. The overall and disease-free survival at 5 years was 59% and 64%, respectively, which decreased to 33% and 39%, respectively, by 10 years. Sarmiento *et al*[2] reported 13 PPD patients in the Mayo Clinic over a 25-year history. Mean follow-up was 6.7 years. Eleven patients underwent local resection without adjuvant therapy. Almost all recurrences were treated by

wider local excision. The 5-year recurrence rate was 61%. Overall 5-year and 10-year survival was 67%. Marchesa *et al*[1] reviewed their experience in the Cleveland Clinic Foundation: ten patients with a mean follow-up longer than 5 years were studied to determine the outcome after surgical treatment. Two patients underwent local excision (LE) with macroscopic clearance of the surgical margins; the remaining eight patients underwent wide local excision (WLE), i.e. > 1 cm microscopic clearance of the surgical margins. The actuarial 8-year recurrence rate for patients treated with LE and WLE was 100% and 50%, respectively. Progression to invasive carcinoma occurred after a median time of 56 mo (range 23-72 mo) in two patients treated with LE, and in one of eight patients treated with WLE. All four patients with recurrence after WLE were successfully treated (no further recurrence) with a second WLE. Actuarial 8-year survival was 0% in the LE group and 40% (SE = 21.9) in the WLE group.

However, as shown above, most of the reports were limited in patient number and to the simple form of PPD. For patients with underlying anorectal cancer, the information was scarce and required documentation. In this study, eight patients with the aggressive subtype were presented, which is the largest group studied to date. One patient died from lung metastases of rectal cancer 10 mo after operation. The other seven cases are tumor-free. This result verified that underlying anorectal cancer was the most important prognostic factor of PPD. Successful management of this lesion is essential for survival.

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COMMENTS

Background

Until now, less than 200 cases of perianal Paget's disease (PPD) had been reported in the literature, most of which were case reports. Unfortunately, frequent misdiagnoses and/or neglect of underlying cancer occurred in the clinical practice. Thus it was necessary to comprehensively document this disease to summarize its clinical and pathological characteristics, to analyze its carcinogenesis, and to explore rational treatment modalities.

Research frontiers

In this study, the authors non-selectively and consecutively documented PPD treated in their institution. All of the cases were exclusively the aggressive subtype with underlying cancer. These clinical experiences provided strong reinforcement of the accumulated knowledge of this disease.

Innovations and breakthroughs

This study well delineated the clinical characteristics of this unusual disease and determined the fundamental modality of preoperative workup, surgery, and postoperative surveillance. In particular, the study demonstrated the mucin-producing character of underlying anorectal cancer and suggested its relationship with carcinogenesis of Paget's disease.

Applications

In this article, the clinical characteristics of PPD and its close relationship with anorectal cancer have been demonstrated. The data is a major contribution to the documentation of this disease and will help clinicians to make correct diagnoses and to choose rational treatment modalities.

Terminology

Paget's disease is a type of cancer with low invasiveness and often occurs in areas rich in sweat gland structures, such as the nipple, axilla, groin, scrotum, and vulva. However, Paget's disease in the perianal region usually has a feature of underlying anal or rectal cancer, is particularly aggressive, and deserves more attention.

Peer review

The authors have to be commended for this very large series of patients with perianal Paget's disease. This manuscript is very interesting and well written.

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BRIEF ARTICLE

CYP2E1 Pst I /Rsa I polymorphism and colorectal cancer risk: A meta-analysis

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Abstract

AIM: To clarify the association between *CYP2E1 Pst I /Rsa I* polymorphism and susceptibility to colorectal cancer.

METHODS: A meta-analysis based on 10 eligible case-control studies involving 4979 cases and 6012 controls was carried out to summarize the data on the association between *CYP2E1 Rsa I /Pst I* polymorphism and colorectal cancer risk.

RESULTS: In comparison of the homozygote *c2c2* and *c2* carriers (*c1c2* + *c2c2*) and the homozygous wild-type genotype (*c1c1*), no association was found between *CYP2E1 Rsa I /Pst I* polymorphism and colorectal cancer risk [odds ratio (OR) = 1.24 (95% CI: 0.93-1.66) for *c2c2*; OR = 1.02 (95% CI: 0.88-1.19) for *c2* carriers]. In stratified analysis, Caucasians with *c2c2* homozygote appeared to have an increased risk of colorectal cancer (OR = 2.67, 95% CI: 1.03-6.89, *P* = 0.043), no significant associations were found in other groups.

CONCLUSION: *c2c2* homozygote of *CYP2E1 Pst I /Rsa I* polymorphism may be associated with the increased risk of colorectal cancer in Caucasians, which needs further investigations.

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Key words: Cytochrome P450 2E1; *Pst I /Rsa I* polymorphism; Colorectal cancer; Cancer susceptibility; Meta-analysis

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INTRODUCTION

Colorectal cancer is one of the most common cancers in developed countries and getting more and more attentions in developing countries for its morbidity and mortality. The definite mechanism of its development is still unknown, but both environmental factors and genetic susceptibility are believed to contribute to the onset of colorectal cancer[1,2].

Cytochrome P450 2E1 (CYP2E1), a member of the cytochrome P-450 superfamily, is a naturally ethanol-inducible enzyme and primarily responsible for the metabolic activation of many low molecular weight carcinogens[3,4], including certain nitrosamines believed to participate in the carcinogenesis of digestive tract[5]. Based on the biological significance of CYP2E1, a hypothesis has been proposed that *CYP2E1* polymorphisms may

be associated with cancer susceptibility[6]. Of the many known genetic polymorphisms in the *CYP2E1* gene, two point mutations in the 5'-flanking region (*Pst I*, *Rsa I*), which are in close linkage disequilibrium, have drawn much interest as it is known to alter the transcriptional activity of the gene[7]. Three genotypes consisting of wild-type allele (*c1*) and variant-type allele (*c2*) of *Pst I* / *Rsa I* polymorphism are defined as homozygous wild-type genotype (*c1c1*), heterozygote (*c1c2*) and rare homozygote (*c2c2*), respectively[8].

Recently, meta-analyses showed that *c2* allele carriers had a significantly lower risk of lung cancer in the Asian population, but a higher risk of gastric cancer. However, the conclusion regarding colorectal cancer remains to be established. Though several studies focusing on *Pst I* / *Rsa I* polymorphism and susceptibility to colorectal cancer have been conducted, the results are inconsistent due to the small sample size of individual study or other factors such as race, diet and tumor location[9-18]. For better understanding this relationship, a meta-analysis was performed.

MATERIALS AND METHODS

Study identification and selection

Inclusion criteria was defined as follows: (1) articles evaluating the association between *CYP2E1 Pst I* /*Rsa I* polymorphism and colorectal cancer risk; (2) studies designed as case-control; (3) sufficient data available to estimate an odds ratio (OR) with its 95% CI.

A literature search of Medline and Embase (updated to January 1, 2010) was conducted using the following terms: 'cytochrome p450 2E1/ⅡE1' or 'CYP2E1/ⅡE1', 'colorectal' and 'cancer' or 'adencarcinoma', without restriction on language. The retrieved literatures were then read in their entirety to assess their appropriateness for the inclusion in this meta-analysis by the two authors (Zhou GW, Hu J) independently. The reference lists of reviews and retrieved articles were searched simultaneously to find additional eligible studies. If more than one article was published by the same author using the same case series, we selected the study with the largest size of samples. If there was an disagreement, it was resolved through discussion between the two authors.

Data extraction

The following variables were extracted from each study if available: first author's surname, publication year, country, ethnicity, source of controls (hospital-based or population-based), matching criteria, number of cases and controls, and number of cases and controls in different *Pst I* / *Rsa I* genotypes.

Statistical analysis

The strength of association between *CYP2E1 Pst I* /*Rsa I* polymorphism and colorectal cancer risk was assessed by odds ratio (OR) with the corresponding 95% CI. The pooled OR was calculated by a fixed-effects model (the Mantel-Haenszel method)[19] or a random-effects model (the DerSimonian and Laird method)[20] according to the heterogeneity. Heterogeneity among studies was checked by the Q test[21] and $P < 0.10$ was considered statistically significant. The ORs of colorectal cancer associated with *c2* allele carriers (*c1c2* + *c2c2*) and homozygous *c2* genotype (*c2c2*) for the *CYP2E1 Pst I* /*Rsa I* polymorphism were estimated using the homozygous wild-type (*c1c1*) as the reference group. Influence analysis was performed by omitting an individual study each time to find potential outliers to the pooled OR[22]; sensitivity analysis was also performed, if necessary, by excluding the Hardy-Weinberg equilibrium (HWE)-violating studies to check the robustness of the result. Departure from HWE was detected in the control populations, but a deviation from HWE was allowed in mixed control populations. Subgroup analyses for different ethnicities (Asian, Caucasian and mixed population) and cancer locations (colon cancer, rectum cancer) were conducted. The possible publication bias was examined visually in a funnel plot of log[OR] against its standard error (SE), and the degree of asymmetry was tested by Egger's test ($P < 0.05$ was considered a significant publication bias)[23]. Meta-analysis was performed using the STATA version 10.0 software.

RESULTS

Study characteristics

A total of 13 publications met the inclusion criteria. Of these studies, two studies[24,25] were excluded as another two included studies[14,16] were based on the same population with a larger sample size. With 14 more cases but less information on the relationship studied in this paper, Chen's study[26] published in 2005 overlapped with Yu's work[11], and it was excluded after discussion. As a result, a total of 10 publications[9-18] containing 4979 cases and 6012 controls were included into this meta-analysis. Table 1 lists the main characteristics of these studies. Of these publications, only one was not published in English but in Chinese[11]. The sample sizes ranged from 419 to 2144. All of the cases were histologically confirmed as colorectal cancer. Controls were mainly healthy populations, and matched with age, sex, or cancer-free. There were three groups of Asians, six groups of Caucasians and one group of mixed populations. Four studies[10,11,16,18] provided information on *Pst I* /*Rsa I* polymorphism status associated with tumor locations classified as colon cancer and rectum cancer. Four studies[10,15,16,18] investigated the significance of *Pst I* /*Rsa I* polymorphism interacting with environmental factors with regard to susceptibility to colorectal cancer. Genotype distributions in the controls of all studies were in agreement with HWE except for one study with mixed control populations[10], without conducting sensitivity analysis by excluding the HWE-violating studies.

Meta-analysis results

Table 2 lists the main results of this meta-analysis. When the homozygote *c2c2* and *c2* carriers (*c2c2* + *c1c2*) were compared with the homozygous wild-type genotype (*c1c1*), the pooled ORs for all the 10 studies were 1.24 (95% CI:

Table 1 Main characteristics of studies included in this meta-analysis

Author, yr	Ethnicity (country)	Source of controls	Matching criteria	Sample size (case/control)	Genotype (case/controls)			HWE
					c1c1	*c2c2*	*c1c2 + c2c2*	
Butler *et al*[9], 2001	Caucasian (Australian)	Population-based	Age, sex	219/200	147/194	NA/NA	2/6	NA
Le Marchand *et al*[10], 2002	Mixed (Japanese, Caucasian, Hawaiian)	Population-based	Age, sex, ethnicity	521/639	384/449	21/26	137/190	No[1]
Yu *et al*[11], 2004	Asian (Chinese)	Population-based	Cancer-free	126/343	69/209	3/8	57/129	Yes
Landi *et al*[12], 2005	Caucasian (Spanish)	Hospital-based	Cancer-free	377/326	323/283	1/1	18/16	Yes
van der Logt *et al*[13], 2006	Caucasian (Netherlander)	Population-based	Age > 18-yr, without (family) history of CRC	371/415	333/389	1/2	24/23	Yes
Kiss *et al*[14], 2007	Caucasian (Hungarian)	Both	Age, sex, red meat intake, smoking	500/500	428/456	7/2	72/44	Yes
Küry *et al*[15], 2007	Caucasian (French)	Population-based	Age, sex	1023/1121	940/1027	6/1	73/91	Yes
Gao *et al*[16], 2007	Asian (Chinese)	Population-based	Age, sex, ethnicity	315/439	185/266	22/13	128/167	Yes
Cotterchio *et al*[17], 2008	Caucasian (Canadian)	Population-based	Age, sex	842/1251	784/1162	0/0	48/85	Yes
Morita *et al*[18], 2009	Asian (Japanese)	Population-based	Sex, smoking, red meat intake, residence area	685/778	412/455	36/44	273/323	Yes

[1]The deviation from HWE was allowed in mixed control populations. HWE: Hardy-Weinberg equilibrium; NA: Not available.

Table 2 Results of meta-analysis for *CYP2E1 Pst I /Rsa I* polymorphism and colorectal cancer risk

Analysis	Number of cases/controls	*c1c2 + c2c2 vs c1c1*		*c2c2 vs c1c1*	
		OR (95% CI)	*P*/P_h	OR (95% CI)	*P*/P_h
Overall	4979/6012	1.02 (0.88, 1.19)	0.780/0.094	1.24 (0.93, 1.66)	0.148/0.148
Subgroup analysis for ethnicity					
Asian	1126/1560	1.03 (0.88, 1.21)	0.707/0.274	1.35 (0.66, 2.75)	0.414/0.072
Caucasian	3332/3813	1.05 (0.78, 1.41)	0.747/0.066	2.67 (1.03, 6.89)	0.043/0.389
Mixed	521/639	0.84 (0.65, 1.09)	0.196/NA	0.94 (0.52, 1.71)	0.850/NA
Subgroup analysis for tumor location					
Colon cancer	907/2188	1.09 (0.83, 1.43)	0.522/0.082	1.22 (0.85, 1.77)	0.279/0.809
Rectum cancer	739/2188	0.89 (0.75, 1.06)	0.206/0.241	0.97 (0.38, 2.43)	0.941/0.014

P_h: *P* values for heterogeneity from *Q* test.

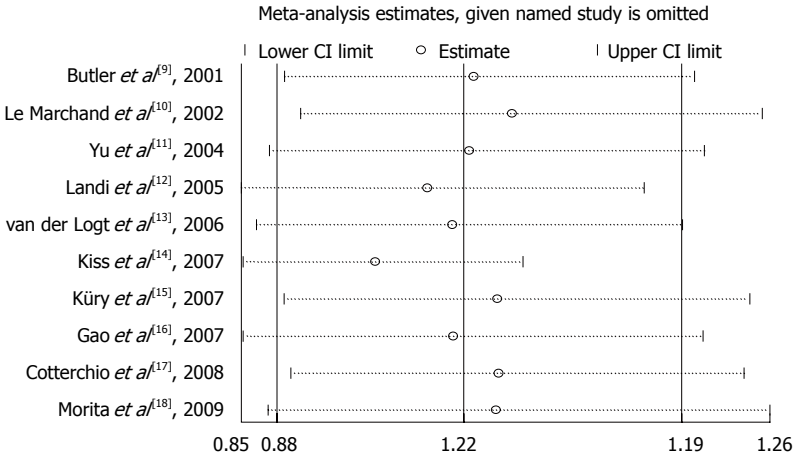


Figure 1 Influence analysis for *c1c2 + c2c2 vs c1c1* in the overall meta-analysis. It shows the influence of individual studies on the summary OR. The middle vertical axis indicates the overall OR and the two vertical axes indicate its 95% CI. Each hollow round indicates the pooled OR when the left study is omitted in this meta-analysis. The two ends of each broken line represent the 95% CI.

0.93-1.66, *P* = 0.148) and 1.02 (95% CI: 0.88-1.19, *P* = 0.780), respectively. In the subgroup analysis of ethnicity, Caucasians with c2c2 homozygote appeared to have an increased risk of colorectal cancer (OR = 2.67, 95% CI: 1.03-6.89, *P* = 0.043), while no significant association was found among Asians and mixed populations. In the subgroup analysis of cancer locations, no significant associations were discovered in both colon cancer and rectum cancer.

Sensitivity analysis

Influence analysis was performed to assess the effects of each individual study on the pooled OR in each analysis, by removing an individual study each time. The results

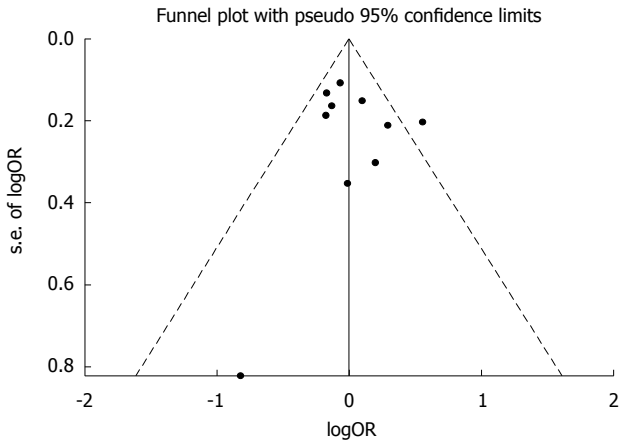


Figure 2 Funnel plot of *CYP2E1 Pst I /Rsa I* polymorphism and colorectal cancer risk for publication bias.

suggested that no individual study significantly affected the pooled ORs. Figure 1 shows the result of influence analysis for *c1c2 + c2c2 vs c1c1*.

Publication bias

Funnel plot and Egger's test were performed to assess the publication bias. The shape of the funnel plot did not indicate any evidence of obvious asymmetry (Figure 2) and no publication bias was found in the Egger's test $P = 0.647$).

DISCUSSION

The relationship between *CYP2E1 Pst I /Rsa I* polymorphism and colorectal cancer susceptibility has been studied in several researches[9-18], and the results are controversial. Kiss and his colleagues[14] reported that *c2* variant allele significantly increased the risk of colorectal cancer, while Gao *et al*[16] suggested that only *c2c2* homozygote was associated with colorectal cancer, and no significant association was found in other studies[10,13]. This is the first systematic study of the meta-analysis of this relationship.

Ten case-control studies were included in this meta-analysis, involving 4979 cases and 6012 controls. The results strongly suggested that there was no significant association between *CYP2E1 Pst I /Rsa I* polymorphism and colorectal cancer susceptibility. By sequentially removing individual studies during influence analysis, it was confirmed that all results were not materially altered by an individual study. In addition, no significant publication bias was found.

Analysis based on the ethnic subgroup showed that Caucasians with *c2c2* homozygote had an increased risk of colorectal cancer. This may be explained by various frequency distributions of *Pst I /Rsa I c2* allele[27] and different living habits and environment in different ethnicity. However, no study regarding African populations was done.

Sensitivity of colon and rectum tissue to carcinogens is inconsistent. In contrast to studies involving rectal cancer, animal studies suggested that nitrosamines may have no correlation with the development of colon cancer[28]. Based on this phenomenon, it is proposed that differences may exist between colon and rectal cancer susceptibility with respect to *Pst I /Rsa I* polymorphism. Morita *et al*[18] found that *c2* allele may significantly reduce the risk of rectal cancer, while in a similar study, Gao's results[16] were completely the opposite. On the other hand, no association was revealed between *c2* allele and colon cancer in these two studies. But Yu *et al*[11] suggested that *c2* allele may increase the susceptibility to colon cancer. In this meta-analysis, a stratified analysis was performed by tumor location due to these conflicting results, and no association was revealed in neither the colon cancer group nor the rectal cancer group (Table 2). A very noteworthy point was that subjects investigated in this stratified analysis were mainly Asians, and studies on Caucasians are needed.

Although the result of this meta-analysis is suggestive, some limitations still exist. Firstly, heterogeneity among the studies, resulting from different defined controls or some other factors, may influence the results of this analysis. In some studies, the controls were selected randomly from a healthy population, and in other studies from hospital-based cancer-free patients. Furthermore, the matching criteria of the control group differed across studies in age and gender. The variant risks of colorectal cancer in these different populations may affect the results. Secondly, OR value was obtained without correction. More accurate OR should be corrected by age, gender and other factors. Thirdly, no African population was included in these ten studies, and the frequency distribution of *CYP2E1 Pst I /Rsa I* genotype in Africans was significantly different from that in Caucasians and Asians[27]. Therefore, the conclusion of this study is not applicable to the African population, and further investigations are required. Fourthly, the relationship between *CYP2E1* gene polymorphism and colorectal cancer risk was analyzed without consideration of gene-environment interactions. Although evidence supported that *c2* allele combined with smoking, drinking and consumption of red meat intake can significantly increase the susceptibility to colorectal cancer[10,15,16,18], this study failed to draw any concrete conclusions due to the different classification criteria of environmental factors in these studies. In addition, there has been no study on the association between *Pst I /Rsa I* polymorphism gene-gene interactions and colorectal cancer risk, and this should be further investigated.

COMMENTS

Background

Cytochrome P450 2E1 (CYP2E1) is a member of the cytochrome P-450 superfamily, and its gene polymorphisms are supposed to be associated with cancer susceptibility. Several studies focusing on *CYP2E1 Pst I /Rsa I* polymorphism and colorectal cancer susceptibility have been conducted.

Research frontiers

The relationship between *CYP2E1 Pst I /Rsa I* polymorphism and cancer susceptibility has been studied in different tumor types. Recently, meta-analyses showed that c2 allele carriers had a significantly lower risk of lung cancer in Asian population but a higher risk of gastric cancer. However, the relationship with colorectal cancer remains controversial and no meta-analysis has been conducted.

Innovations and breakthroughs

This is the first meta-analysis which systemically studied the relationship between *CYP2E1 Pst I /Rsa I* polymorphism and colorectal cancer susceptibility, and suggested that Caucasians with *c2c2* homozygote may have a higher risk of colorectal cancer.

Applications

This study provided a potential biomarker for identifying high-risk populations of colorectal cancer in Caucasians.

Peer review

The article is meritoriously and methodologically well written. The presentation is rigorous and the approach used is robust.

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CASE REPORT

Autoimmune pancreatitis with atypical imaging findings that mimicked an endocrine tumor

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Abstract

Autoimmune pancreatitis (AIP) is a rare cause of recurrent acute pancreatitis or chronic pancreatitis in middle-aged patients, and is characterised by a marked infiltration of lymphocytes and plasma cells in pancreatic tissue. Diagnosis of focal forms can be difficult as AIP may mimic pancreatic adenocarcinoma. Pediatric cases of AIP are exceptional. We report the case of a 15-year-old girl who had a focal AIP and associated cholangitis, with a very unusual vascularized mass that mimicked a pancreatic endocrine tumor. The diagnosis was obtained by a pancreatic biopsy, thus avoiding surgical resection, and all the clinical, biological and radiological abnormalities resolved after steroid therapy with 6 mo of follow-up.

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Key words: Pancreas; Autoimmune pancreatitis; Adolescent; Endocrine tumor; Biopsy

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INTRODUCTION

Autoimmune pancreatitis (AIP) is a rare cause of recurrent acute pancreatitis and chronic pancreatitis. First described by Sarles in 1960, AIP is characterised by a marked infiltration of lymphocytes and plasma cells in pancreatic tissue (lymphoplasmocytic sclerosing pancreatitis)[1-5]. It can be associated with extrapancreatic lesions (including sclerosing cholangitis, retroperitoneal fibrosis, nephritis, sialoadenitis, chronic inflammatory bowel disease) and is the pancreatic manifestation of a systemic inflammatory disease called IgG4-associated systemic disease in about one third of cases[6-9]. Two radiological types are described: a diffuse form (the most frequent, 70% of cases) and a focal form (30% of cases) that may mimic pancreatic adenocarcinoma[7,10,11].

Most cases have been reported in male patients (sex ratio = 3:1), and in patients older than 55 years (average age: 65 years); less than 10 pediatric cases have been reported[9,12-19]. We report the case of a 15-year-old girl who had a focal AIP and associated cholangitis, with atypical clinicoradiological features, that mimicked a pancreatic endocrine tumor.

CASE REPORT

A 15-year-old girl was referred to our unit for the exploration of a pancreatic mass. She had a 1-mo history of epigastric pain and nausea, associated with jaundice and pruritus for 5 d, without either fever or weight loss. She had a personal history of asthma in childhood, and no family medical history. She was not taking any drugs. Over the previous 3 years, she had had several episodes of weakness, with fainting, tremor, sweating and hunger, which resolved after eating.

Examination was normal except for the finding of jaundice. Total bilirubin was 142 μmol/L (conjugated: 104 μmol/L), aspartate aminotransferase was 6 times the upper limit of normal (ULN), alanine aminotransferase was 16 ULN and γ-GT was 4 ULN; blood ionogram results including serum creatinine level, blood cell counts and prothrombin time were normal.

Abdominal ultrasonography showed enlargement of the intrahepatic and common bile ducts (common bile duct diameter: 11 mm), an enlarged gallbladder without either lithiasis or thick wall, and enlargement of the main pancreatic duct due to an oval mass located in the head of the pancreas which was quite well limited, hypoechogenic and homogeneous, measuring 22 mm × 15 mm. Abdominal multi-detector computed tomography (MDCT) revealed a lesion of the head of the pancreas, isodense before intravenous contrast medium injection and that moderately enhanced after injection, measuring 20 mm in diameter, with bicanalar dilation and gallbladder enlargement, without invasion of the duodenum or the vascular structures, and with neither suspicious lymph nodes nor liver metastasis (Figure 1A and B). Bilio-pancreatic magnetic resonance imaging (MRI) showed that the lesion was hypointense in T1-weighted sequences and isointense in T2-weighted compared with surrounding pancreatic parenchyma, and enhanced after intravenous contrast medium injection in arterial and portal venous phases; the main pancreatic duct was enlarged, irregular, and the terminal part of the common bile duct had a thick enhanced wall suggesting cholangitis (Figure 2A and B).

Because the mass was hypervascular on imaging and the patient reported episodes of weakness suggesting hypoglycemia, we considered the diagnosis of pancreatic insulinoma. A 72-h fast test and the blood levels of insulin and C-peptide were normal. Chromogranin A and neuron specific enolase levels were also normal.

Due to all these atypical features, we preferred performing a pancreatic biopsy rather than a somatostatin-receptor scintigraphy at this stage of investigation. Endoscopic ultrasonography (EUS) showed a heterogeneous

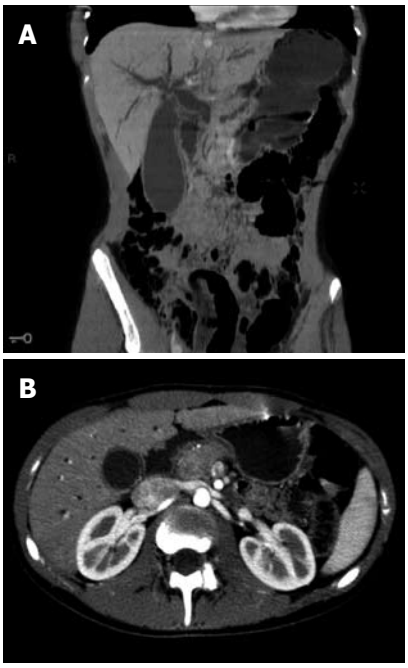


Figure 1 Initial multi-detector computed tomography scan imaging. A: CT scan before contrast injection, coronal reconstruction: enlargement of the main pancreatic duct and the biliary ducts, with enlarged gallbladder; B: CT scan after contrast injection, arterial phase, axial slice: lesion of the head of the pancreas, moderately enhanced after contrast injection.

area in the head of the pancreas measuring 23 mm × 35 mm. The main pancreatic duct was much enlarged (maximal diameter: 6 mm) along its whole length, with a moniliform aspect; the accessory pancreatic duct was also abnormally visible. The terminal part of the common bile duct had a thick wall. There was a narrowing of the terminal part of the superior mesenteric vein, without invasion of its wall. All these features were not evocative of a malignant tumor. The fine needle aspiration specimen was moderately cellular and consisted of cellular stromal fragments in a mesenchymal and lymphocytic background. At higher magnification, the stromal fragments contained bland spindled mesenchymal cells with a lymphocytic infiltrate. Rare plasma cells were also present in the stromal fragments. No granulomas were seen. There were no malignant cells. Histological examination of the duodenal biopsy showed normal mucosa with scattered plasma cells; examination of the adjacent pancreatic parenchyma revealed chronic pancreatitis with atrophic acini surrounded by a fibrous stroma, associated with periductal lymphoplasmocytic infiltration and obliterative phlebitis (Figure 3A and B). The plasma cells expressed IgG4 (Figure 3C and D).

Based on these results, the diagnosis of focal AIP associated with cholangitis was proposed. IgG4 serum level was normal (100 mg/mL, N < 140 mg/mL) and the antinuclear antibody test was negative (< 1/80).

Jaundice decreased spontaneously 1 wk after admission (total bilirubin: 69 μmol/L; conjugated bilirubin: 50 μmol/L). As there was remaining abdominal pain and pruritis, steroid therapy was started at the dose of 40 mg of oral prednisone per day. After 2 wk of treat-

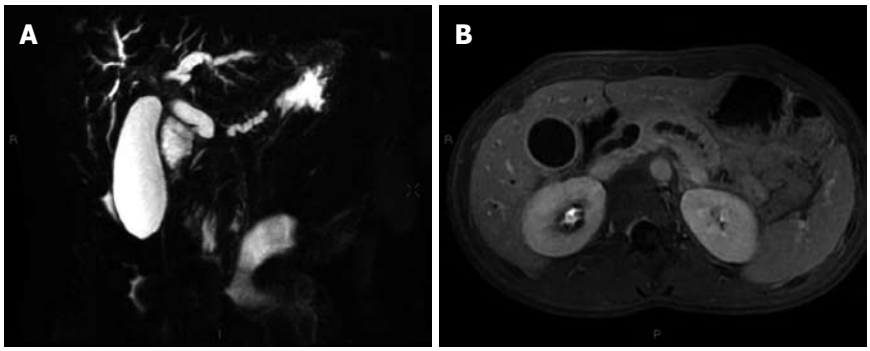


Figure 2 Initial magnetic resonance imaging (MRI) imaging. A: T2-weighted MRI, coronal slice: bicanalar dilation and enlarged gallbladder; B: T1-weighted MRI, axial slice: main biliary duct and gallbladder enlargement with a thick wall evocative of cholangitis; the main pancreatic duct is also irregularly enlarged.

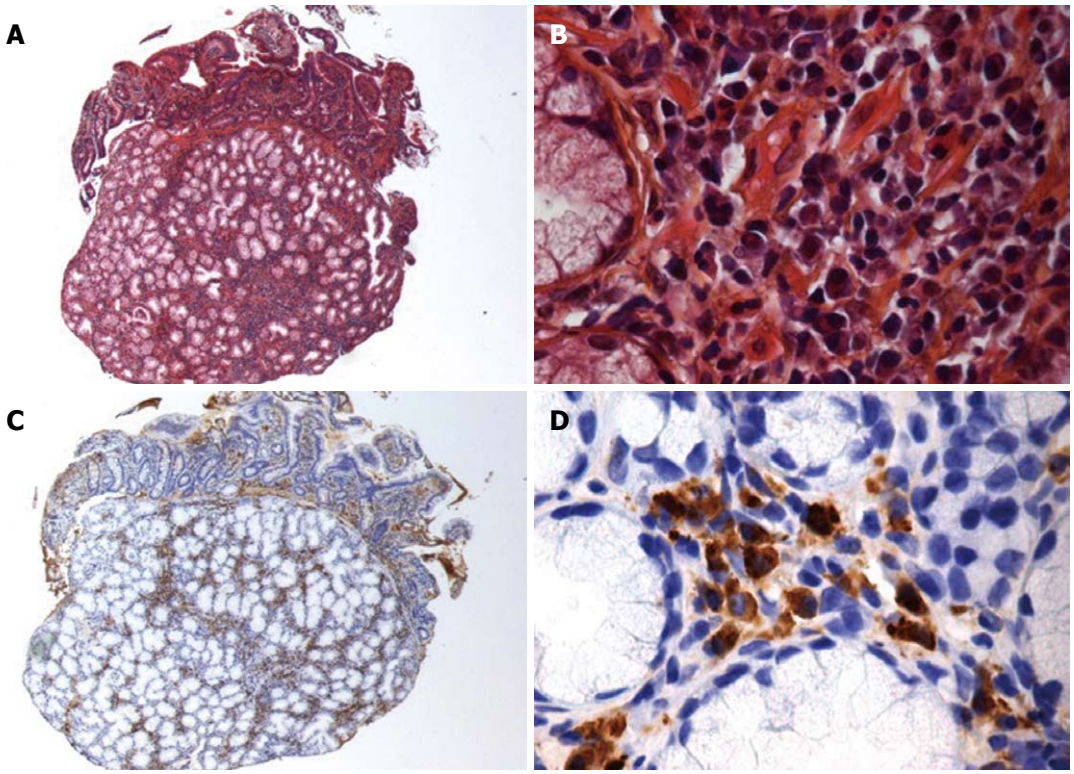


Figure 3 Histological examination of periampular biopsies (notice that both duodenal mucosa and pancreatic parenchyma are visible). A, B: Hematoxylin-eosin staining (A: × 125, B: × 500): atrophic acini surrounded by a fibrous stroma, associated with periductal lymphoplasmocytic infiltration; C, D: Anti-IgG4 immunostaining (C: × 125, D: × 500): the plasma cell infiltration expresses IgG4.

ment, the patient was asymptomatic and hepatic blood tests had returned to normal values. The steroid therapy was continued at the same dose for two additional weeks and progressively decreased and stopped after 3 mo. Bilio-pancreatic MRI at the end of the treatment was normal (Figure 4). Six months after the end of the steroid therapy, the patient remained asymptomatic and the blood tests were normal.

DISCUSSION

Most cases of AIP occur in middle-aged patients and about half of the patients are affected when in their sixties[9,12,13]. Cases occurring in childhood or adolescence are exceptional: to our knowledge, only five pediatric cases have been reported (range: 10-16 years), including one in association with a hereditary chronic pancreatitis[14-19].

The most frequent morphological aspect of AIP is the diffuse "duct destructive" form (70% of cases): the pancreas is diffusely enlarged and a loss of lobularity is commonly seen ("sausage-like" appearance); the swollen parenchyma is hypodense on MDCT, hypointense on MRI in T1-weighted sequences and there is a delayed and moderate enhancement after intravenous contrast medium injection due to fibrosis[7,11,20,21]. A typical capsule-like rim appearing as a low-density area on MDCT and a hypointense T2 area on MRI surrounding the pancreas is often present[20,21]. The main pancreatic duct is diffusely narrowed, irregular, or even not visible, because of the pericanalicular inflammatory infiltration[20,21]. Pancreatic atrophy and calcifications are rare but have been reported in some chronic cases[7,11,20,21]. In the focal (pseudo-tumoral) form, the lesion typically appears as a hypovascular mass compared with surrounding pancreatic parenchyma: isodense on MDCT and hypointense on MRI in T1-weighted sequences, with a low enhance-



Figure 4 MRI at the end of steroid therapy. T1-weighted MRI with gadolinium injection: normal main pancreatic duct and gallbladder; the mass in the head of the pancreas has totally disappeared.

ment after intravenous contrast medium injection[7,11,21]. This imaging presentation may be difficult to differentiate from a pancreatic adenocarcinoma in a middle-aged patient with pancreatic symptoms[10,11,21,22].

Atypical imaging in patients with AIP is not uncommon; diverse imaging features have been reported. However, this report describes enhancement suggestive of a vascular lesion which has not been described before in AIP and which was suggestive of an endocrine tumor[23]. This could be due to the diagnosis being performed at an early stage thus the pancreatic parenchyma mostly contained acute inflammatory changes and minimal fibrosis. Another noticeable feature was major enlargement of the main pancreatic duct, which was suggestive of an upstream expansion due to a malignant tumor[21]. An upstream pancreatic duct dilation was reported in up to 57% of patients with focal AIP in an earlier study[24]. Similar main pancreatic duct abnormalities have recently been reported in a 14-year-old girl with AIP[19]. Again, this may have resulted from the association of an early stage diagnosis of the AIP and its focal location: pericanalicular fibro-inflammatory infiltration may not have been present at this stage and the pancreatic duct would have been free to enlarge in its distal part upstream from the stenosis due to the extrinsic compression exerted by the mass located in the head of the pancreas. The vascular narrowing in our case was also evocative of malignancy, but this has already been reported in AIP[11,21]. In contrast, the features of cholangitis supported the diagnosis of AIP since IgG4-associated cholangitis is a common extrapancreatic lesion associated with AIP[8,9,21]. Due to the difficulty of differential diagnosis, we preferred performing a pancreatic biopsy rather than a somatostatin-receptor scintigraphy.

Hence, our report underlines the helpfulness of performing pancreatic biopsy for the diagnosis of a pancreatic mass in the case of negative IgG4 serology and atypical imaging findings according to the HISORt criteria, to avoid an unjustified pancreatic resection[25,26]. From our observations, the histologic features were characteristic of lymphoplasmocytic sclerosing pancreatitis[2,5,9]. The positivity of the IgG4 immunostaining also reinforced the suspicion of AIP, despite the fact that the sensitivity of this technique for the diagnosis of AIP is only 21% to 47% in the literature[27,28]. The absence of malignant cells was also helpful in the diagnostic discussion[22]. Several studies have shown the usefulness of the EUS controlled fine-needle biopsy for the diagnosis of AIP[29-32].

Another original element in this observation was the demonstration of IgG4 plasma cell expression by immunostaining in an extrapancreatic location, i.e. duodenal site. Deheragoda *et al*[33] reported that 85% of biopsies in any organs (liver, stomach, duodenum, colon, kidney, bone marrow, salivary gland) from AIP patients exhibited positive IgG4 immunostaining compared with 0.6% of specimens from controls, a result which was independent from the IgG4 serum concentration. In our observation, the presence of IgG4 positive plasma cells outside of the pancreas (periampulla duodenal mucosa) was contributive to the diagnosis, as previously reported[34]. Altogether, these findings support the hypothesis that AIP is likely be a part of a multisystemic disease.

Whereas the common differential diagnosis of focal AIP in adults is pancreatic adenocarcinoma, the originality of our report is that the mass was evocative of endocrine tumor because of its hypervascular aspect on imaging[10,11,22,23]. Pediatric cases of endocrine tumors have been described: the Surveillance, Epidemiology, and End Results (SEER) register of the National Cancer Institute registered 19 cases between 1973 and 2004 in North America[35]. Jaundice is very uncommon in endocrine tumors (< 5% of cases), whereas about one third (up to 70%-80%) of AIP patients have jaundice[13,36]. The pancreatic biopsy examination and the favorable evolution before and after steroid therapy allowed us to rule out other differential diagnoses such as a solid-pseudopapillary tumor, a pancreatoblastoma (10 cases each reported in the SEER register) or other malignant pancreatic tumors (canalar adenocarcinoma, sarcoma and acinar adenocarcinoma: 7, 4 and 5 cases, respectively in the SEER register)[3,6,35]. Overall, pancreatic biopsies may be useful to avoid misdiagnosing and delaying the treatment of a malignant pancreatic lesion in pediatric cases[10,11,22,37].

The particular problem in pediatric cases of AIP is the risk of pancreatic insufficiency due to recurrence and pancreatic atrophy, as well as extrapancreatic involvement with occurrence of other autoimmune disorders, such as chronic inflammatory bowel disease[7,9,11,38-40]. Consequently, it is important to carefully follow up these young patients in order to diagnose early and treat these complications, paying particular attention to steroid therapy side effects.

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CASE REPORT

Acute necrotizing pancreatitis as first manifestation of primary hyperparathyroidism

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Abstract

We report the case of a female patient with severe acute necrotizing pancreatitis associated with hypercalcemia as first manifestation of primary hyperparathyroidism caused by a benign parathyroid adenoma. Initially the acute pancreatitis was treated conservatively. The patient subsequently underwent surgical resection of the parathyroid adenoma and surgical clearance of a large infected pancreatic pseudocyst. Although the association of parathyroid adenoma-induced hypercalcemia and acute pancreatitis is a known medical entity, it is very uncommon. The pathophysiology of hypercalcemia-induced acute pancreatitis is therefore not well known, although some mechanisms have been proposed. It is important to treat the provoking factor. Therefore, the cause of hypercalcemia should be identified early. Surgical resection of the parathyroid adenoma is the ultimate therapy.

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Key words: Acute necrotizing pancreatitis; Hypercalcemia; Primary hyperparathyroidism; Parathyroid adenoma

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INTRODUCTION

Acute necrotizing pancreatitis is a severe condition with a high mortality rate[1]. In the Western world, alcohol and biliary stones are the main causes of acute pancreatitis[2,3]. As shown by the Ranson[4] grading of acute non-alcoholic pancreatitis, severe pancreatitis is associated with a decrease in serum calcium levels. However, the relationship between increased serum calcium levels and acute pancreatitis may be a diagnostic clue to primary hyperparathyroidism (PHPT)[5]. Acute pancreatitis caused by hypercalcemia due to PHPT is a very rare condition[6]. We present a case report of a young female patient with hypercalcemia-induced acute necrotizing pancreatitis as first manifestation of a benign parathyroid adenoma.

CASE REPORT

A 42-year-old female presented at the emergency room with a sudden attack of severe epigastric pain and vomiting. Years ago she had undergone surgery for a benign ovarian cyst and a right nephrectomy for ureterolithiasis-

induced hydronephrosis. Her medical history also mentioned three episodes of deep vein thrombosis in the lower limb.

She was a non-smoker and consumed alcohol only sporadically. There was no recent use of alcohol. She did not take medication and did not travel abroad. Family history mentioned a brother infected with hepatitis B virus.

Clinical examination revealed a severely ill patient, a silent abdomen which was distended with painful percussion and palpation of the epigastrium.

Blood analysis showed signs of inflammation with an elevated C-reactive protein (CRP) of 6.77 mg/dL (< 0.5 mg/dL) and leukocytosis of 30.8 × 10E9/L (4.3-10 × 10E9/L) with elevated neutrophils of 28.1 × 10E9/L (2.0-7.0 × 10E9/L). Pancreatic enzymes were elevated, serum amylase was 1033 U/L (24-72 U/L) and serum lipase was 7189 U/L (13-300 U/L). There was a slight impairment of renal function as shown by an increase in serum creatinine 1.23 mg/dL (0.6-1.1 mg/dL). Both liver transaminases and canalicular liver tests were normal. In contrast to what was expected in a case of severe pancreatitis, serum calcium was increased to 11.7 mg/dL (8.5-10.2 mg/dL), the albumin-modified serum calcium concentration was 11.9 mg/dL. IgG4 was normal as were triglycerides.

Based on the clinical picture and blood analysis she was diagnosed with acute pancreatitis. Abdominal ultrasound was carried out and showed no evidence of cholelithiasis or dilated bile ducts. Abdominal computed tomography (CT) scan confirmed the diagnosis of exudative pancreatitis (Figure 1A). The patient was admitted to the ward and was treated conservatively with intravenous fluids and analgesia. Because of slow improvement of paralytic ileus, enteral feeding was started through a nasojejunal tube. The patient improved gradually during the following weeks. At day 20 after admission, abdominal CT scan was repeated and showed the development of pancreatic pseudocysts and necrosis of the pancreas with few remnants of normal pancreatic tissue (Figure 1B).

After exclusion of alcohol-induced and biliary pancreatitis as well as auto-immune pancreatitis, elevated serum calcium levels remained another potential etiology of acute pancreatitis. On reviewing her medical records, normal serum calcium levels at the time of previous surgical interventions was noted. Serum levels of intact parathyroid hormone (iPTH) were determined and showed an increase up to 82 pg/mL (13-54 pg/mL). This result confirmed hypercalcemia caused by hyperparathyroidism. Ultrasound of the parathyroid glands showed no abnormalities. A 99mTc-Sestamibi scintigraphy was then carried out which was consistent with a lower left side parathyroid adenoma (Figure 2).

The patient underwent surgical resection of this tumor, and histological examination confirmed the diagnosis of benign parathyroid adenoma. In the postoperative period, serum levels of iPTH and calcium decreased and the patient required calcium supplements. Following clinical resolution, the patient was discharged from hospital.

A few weeks later she was re-admitted to the emergency room due to abdominal pain and fever. Blood analysis showed elevated CRP of 15.18 mg/dL (< 0.5 mg/dL), serum amylase and serum lipase were normal. Serum calcium was slightly decreased: 8.3 mg/dL (8.5-10.2 mg/dL). Infection of the previously diagnosed pancreatic pseudocysts was suspected. Abdominal magnetic resonance imaging (MRI) and endoscopic ultrasonography showed debris in the large pseudocyst (Figure 3). Initially she was treated with broad spectrum antibiotics, however, she suffered recurrent episodes of fever and abdominal pain. Abdominal CT scan revealed rupture of the largest pancreatic pseudocyst. Surgical cystogastrostomy and necrosectomy of the pancreatic corpus was performed. She recovered well and the patient remained free of symptoms without signs of exocrine or endocrine pancreas insufficiency.

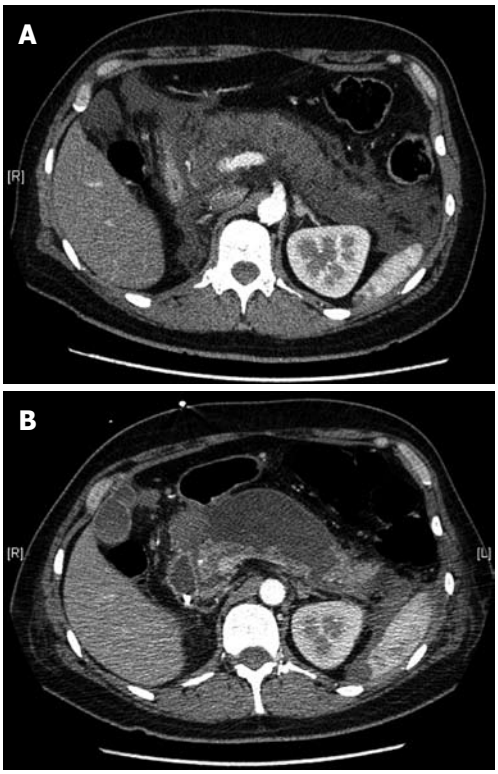


Figure 1 Abdominal computed tomography scan at the level of the pancreas. A: On the day of admission showing acute exudative pancreatitis. Note the history of a right nephrectomy; B: On day 20 showing the development of pancreatic pseudocysts and few remnants of normal pancreatic tissue.

DISCUSSION

Acute pancreatitis caused by PHPT-induced hypercalcemia is a rare condition. It was first described in 1957 by Cope *et al*[5]. Since then, the relationship between PHPT and pancreatitis has been questioned, but nowadays PHPT has been acknowledged as an accepted etiology of pancreatitis[1,7,8]. The prevalence of acute pancreatitis in patients with PHPT is estimated between 1.5% and 7%[6]. Some studies have associated hyperparathyroidism with pancreatitis, however, the prevalence of acute pancreatitis in patients with PHPT seems no different from that in the general population[9,10]. Therefore, there appears

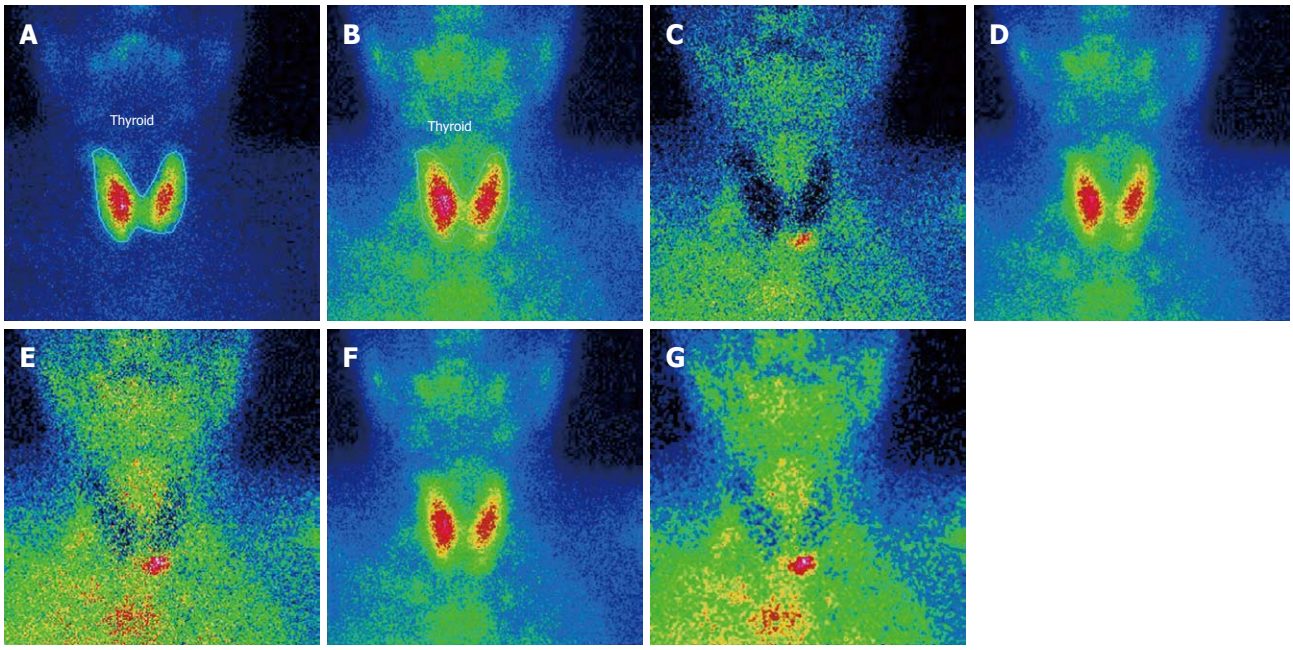


Figure 2 99mTc-Sestamibi scintigraphy showing a lower left side parathyroid adenoma. A: Thyroid SKM: SK ELUAAT, -Tc-99mANT; B: Parathyroid SKM: SK MIBI, -Tc-99mH1; C: Normalized subtracted f = 1; D: Registered parathyroid; E: Normalized subtracted f = 1.25; F: Normalized parathyroid f = 1.25; G: Smoothed subtracted f = 1.25.

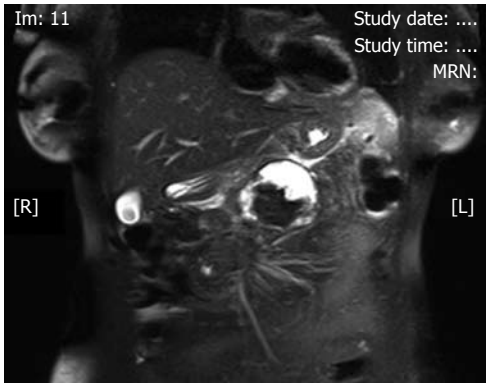


Figure 3 Abdominal magnetic resonance imaging at the level of the pancreas showing debris in the pancreatic pseudocyst.

to be no direct causal relationship between PHPT and acute pancreatitis based on epidemiological data. However, it has been shown that hypercalcemia of any cause can lead to acute pancreatitis[11]. When this combination occurs, pancreatitis is likely to be severe and the degree of hypercalcemia may play an important role in this association[12,13]. Three pathophysiological mechanisms are suggested. The deposition of calcium in the pancreatic duct may cause pancreatic duct obstruction[14]. Hypercalcemia may also lead to activation of trypsinogen within the pancreatic parenchyma causing autodigestion of the pancreas[15]. Finally, genetic variants in SPINK 1 (serine protease inhibitor Kazal type 1) and CFTR (cystic fibrosis transmembrane conductance regulator) genes in combination with hypercalcemia increase the risk of developing acute pancreatitis in patients with PHPT[16].

Usually, acute pancreatitis is associated with a decrease in serum calcium levels. Based on the Ranson[4] grading, low serum calcium levels have prognostic importance. Therefore, it is uncommon to detect hypercalcemia in a patient presenting with severe acute pancreatitis. This unusual condition should always alert physicians to the presence of hyperparathyroidism or malignancy[17,18]. In order to complete the diagnosis, parathyroid hormone levels should be determined and imaging of the parathyroid glands is important. It is also important to take into account the possibility of an ectopic localization of a parathyroid adenoma as a cause of acute pancreatitis[19,20]. In the present case, diagnosis of the parathyroid tumor was made by 99mTc-Sestamibi scintigraphy. Surgical resection and histological examination of the tumor is the ultimate therapy and parathyroidectomy may prevent the recurrence of pancreatitis[7,12]. Subsequent rapid hypocalcemia may develop requiring calcium supplementation. Good cooperation between gastroenterologists, endocrinologists and surgeons is important in treating this rare phenomenon of acute necrotizing pancreatitis caused by PHPT-induced hypercalcemia.

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Meetings

Events Calendar 2010

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Tamilnadu, India
International Conference on Medical Negligence and Litigation in Medical Practice

January 25-29
Waikoloa, HI, United States
Selected Topics in Internal Medicine

January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology Conference

January 28-30
Hong Kong, China
The 1st International Congress on Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of Gastroenterology & Endoscopy Meeting

March 09-12
Brussels, Belgium
30th International Symposium on Intensive Care and Emergency Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian National Association for Study of the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on Diabetes PACD14

March 25-28
Beijing, China
The 20th Conference of the Asian Pacific Association for the Study of the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology and Hepatology Conference, EGHC 2010

April 14-17
Landover, Maryland, United States
12th World Congress of Endoscopic Surgery

April 14-18
Vienna, Austria
The International Liver Congress™ 2010

April 28-May 01
Dubrovnik, Croatia
3rd Central European Congress of surgery and the 5th Croatian Congress of Surgery

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual Meeting

May 06-08
Munich, Germany
The Power of Programming: International Conference on Developmental Origins of Health and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and Rectal Surgeons Annual Meeting

June 04-06
Chicago, IL, United States
American Society of Clinical Oncologists Annual Meeting

June 09-12
Singapore, Singapore
13th International Conference on Emergency Medicine

June 14
Kosice, Slovakia
Gastro-intestinal Models in the Research of Probiotics and Prebiotics-Scientific Symposium

June 16-19
Hong Kong, China
ILTS: International Liver Transplantation Society ILTS Annual International Congress

June 20-23
Mannheim, Germany
16th World Congress for Bronchoesophagology-WCBE

June 25-29
Orlando, FL, United States
70th ADA Diabetes Scientific Sessions

August 28-31
Boston, Massachusetts, United States
10th OESO World Congress on Diseases of the Oesophagus 2010

September 10-12
Montreal, Canada
International Liver Association's Fourth Annual Conference

September 11-12
La Jolla, CA, United States
New Advances in Inflammatory Bowel Disease

September 12-15
Boston, MA, United States
ICAAC: Interscience Conference on Antimicrobial Agents and Chemotherapy Annual Meeting

September 16-18
Prague, Czech Republic
Prague Hepatology Meeting 2010

September 23-26
Prague, Czech Republic
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The 7th Biannual International Symposium of Society of Coloproctology

October 15-20
San Antonio, TX, United States
ACG 2010: American College of Gastroenterology Annual Scienitfic Meeting

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Boston, Massachusetts, United States
The Liver Meeting® 2010--AASLD's 61st Annual Meeting

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December 02-04
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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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4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

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5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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Issue with no volume

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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

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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.

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EDITORIAL

Antiproliferative effect of somatostatin analogs in gastroenteropancreatic neuroendocrine tumors

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Abstract

Somatostatin analogs were initially developed for the control of hormonal syndromes associated with neuroendocrine tumors (NETs). In recent years, accumulating data has supported their role as antiproliferative agents, capable of stabilizing tumor growth in patients with metastatic neuroendocrine malignancies, including carcinoid and pancreatic endocrine tumors. A phase Ⅲ, randomized, placebo-controlled trial has now demonstrated that octreotide long-acting repeatable (LAR) 30 mg can significantly prolong time to tumor progression among patients with metastatic midgut NETs regardless of functional status, chromogranin A level or age. In addition to significantly lengthening time to tumor progression in the overall study population, subset analysis suggests that patients with low tumor burden are most likely to experience disease stabilization with octreotide LAR 30 mg, supporting the early use of octreotide LAR in patients with metastatic disease. Further research efforts are underway to evaluate the use of somatostatin analogs as antiproliferative agents in other types of gastroenteropancreatic-NETs. Ongoing studies are also evaluating novel somatostatin analogs and somatostatin analogs in combination with other anti-tumor therapies.

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INTRODUCTION

The human hormone somatostatin was first isolated in 1973 and identified as a hypothalamic inhibitor of growth hormone[1-4]. It was subsequently discovered in multiple tissues, including the central nervous system, endocrine system and gastrointestinal tract[3]. Somatostatin has been characterized as a universal endocrine "off-switch" due to its exocrine, endocrine, paracrine and autocrine inhibitory effects[5-7]. In the digestive tract, it reduces secretion and motility, decreases portal blood flow, inhibits gallbladder contraction and reduces the secretion of other gastrointestinal hormones[8]. The effects of somatostatin are mediated through interaction with five somatostatin receptors (sst_{1-5})[9], belonging to a family of G-protein coupled receptors with seven transmembrane domains.

The clinical utility of native human somatostatin is limited by its short half life of approximately two minutes. Both bioactive forms of the hormone, the fourteen-peptide somatostatin-14 and a C-terminally extended form, somatostatin-28, contain multiple enzymatic cleavage sites resulting in rapid circulatory degradation[6]. In order to improve the pharmacokinetic profile, synthetic somatostatin

analogs (SSAs) have been developed by shortening the polypeptide chain while retaining binding affinity to somatostatin receptors (Figure 1)[10]. The two commercially available analogs, octreotide and lanreotide, are octapeptides that bind with high affinity to somatostatin receptor subtype 2 (sst_2) and with moderate affinity to sst_5 (Table 1).

Octreotide has been used in clinical practice since data emerged in the 1980s confirming its ability to palliate carcinoid syndrome[14], as well as other hormonal syndromes caused by metastatic gastroenteropancreatic neuroendocrine tumors (GEP-NETs). Octreotide was initially available in an immediate-release formulation suitable for deep subcutaneous or intravenous administration[15]. Octreotide subcutaneous (sc) has been tested primarily at doses ranging from 100 to 500 μg two to three times daily. During the past decade, a long-acting repeatable (LAR) depot formulation of octreotide (Sandostatin LAR®) has been available, which allows monthly intramuscular dosing. Octreotide LAR has demonstrated similar efficacy to octreotide sc in the control of flushing and diarrhea associated with carcinoid syndrome[16]. The dose of octreotide sc and octreotide LAR should be titrated per symptom control for optimal patient care[17]. A second somatostatin analog, lanreotide, was licensed in Europe in 1998 for the treatment of symptoms associated with neuroendocrine (particularly carcinoid) tumors. A long-acting formulation of lanreotide (Somatuline Autogel®)[18] has also been developed as a deep subcutaneous injection.

Early on, clinical trials of SSAs tested their ability to inhibit the release of neuroendocrine hormones such as serotonin, glucagon, insulin, gastrin and vasoactive intestinal peptide (VIP)[14,19-22]. These trials formed the basis for the approval of octreotide and lanreotide as antisecretory agents indicated for treatment of hormonally active GEP-NETs. It was not until several years after the approval of octreotide that evidence of antineoplastic activity emerged. Although objective radiographic responses associated with SSAs were rare, many cases of prolonged disease stability were documented in the literature, leading to the hypothesis that SSAs exert an inhibitory effect on tumor growth. Recently, this hypothesis was tested in a Phase Ⅲ, randomized, placebo-controlled clinical trial evaluating octreotide LAR 30 mg. This review summarizes the preclinical and clinical evidence supporting the role of SSAs as antiproliferative agents in the treatment of patients with GEP-NETs. To date, most data (including the results from the only phase Ⅲ randomized, placebo-controlled trial) have been generated in studies evaluating octreotide sc and LAR.

BIOLOGICAL BASIS FOR THE ANTIPROLIFERATIVE EFFECTS OF SSAS

Over the past two decades there has been significant progress in our understanding of the molecular basis for the antiproliferative effects of somatostatin and its analogs. Antitumoral activity appears to be mediated *via* direct and indirect mechanisms. Direct mechanisms involve the activation of somatostatin receptors on tumor cells leading to modulation of intracellular signaling transduction pathways. Multiple *in vitro* studies using cell lines transfected with somatostatin receptors indicate that all receptor subtypes (sst_{1-5}) may mediate inhibition of cell proliferation[23], whereas specific receptor subtypes ($sst_{2,3}$) may mediate apoptosis (Table 2)[24-26]. These actions appear to be regulated primarily *via* the MAP-kinase signaling pathway and through activation of phosphotyrosine phosphatases (Figure 2)[27-29]. Indirect antiproliferative mechanisms include inhibition of mitogenic growth factors such as insulin-like growth factor (IGF), as well as inhibition of tumor angiogenesis through interaction with somatostatin receptors on endothelial cells and monocytes[30].

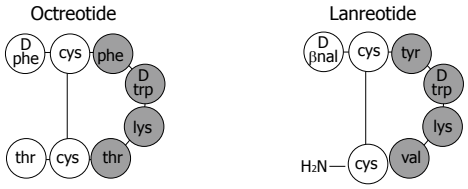


Figure 1 Chemical structure of the synthetic somatostatin analogs octreotide and lanreotide (adapted from[11]).

Table 1 Receptor binding affinities of somatostatin, octreotide and lanreotide

	sst_1	sst_2	sst_3	sst_4	sst_5
Receptor binding affinity (IC_{50} nmol/L)					
Somatostatin[12]	0.93	0.15	0.56	1.50	0.29
Octreotide[12]	280.00	0.38	7.10	> 1000	6.30
Lanreotide[13]	> 1000	0.80	107	> 1000	5.20

Table 2 Receptor mediation of cell proliferation

	sst_1	sst_2	sst_3	sst_4	sst_5
Induction of G1 cell cycle arrest	+	+		+	+
Induction of apoptosis		+	+		

Activation of phosphotyrosine phosphatases

Several phosphotyrosine phosphatases (PTPs), including SHP-1 and SHP-2, have emerged as important regulators of intracellular signaling pathways[27]. Somatostatin receptor-mediated activation of SHP-1 results in arrest of cell proliferation in various cell lines, including cells derived from pancreatic, breast and prostate carcinomas[31,32]. In pituitary adenoma cells, activation of sst_2 inhibits PI3 kinase activity and causes cell growth arrest *via* stimulation of SHP-1[33]. The enzymatic activity of SHP-1 has also been implicated in sst_3-dependent apoptosis in transfected Chinese Hamster Ovary (CHO) cells[34]. Stimulation of SHP-1 in sst_2-expressing CHO cells has led to G1 cell cycle arrest *via* induction of the cyclin-dependent kinase inhibitor p27[35]. SHP-2 has also been identified as a mediator of the antiproliferative effects of somatostatin receptors,

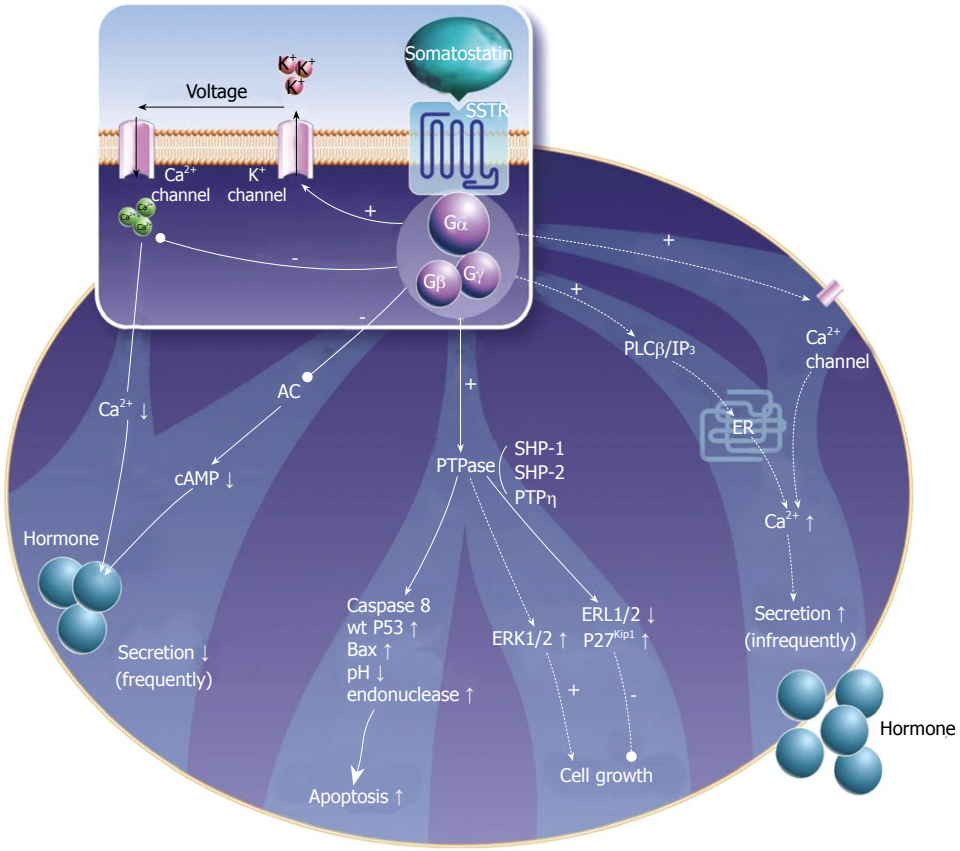


Figure 2 Somatostatin receptor-mediated effects on neuroendocrine cells (adapted from[23]).

primarily through inactivation of tyrosine kinase receptors for insulin and epidermal growth factors[36]. Moreover, activation of PTPs has been shown to down-regulate Raf-1[37] and block the MAP-kinase pathway[38].

Modulation of the mitogen activated protein-kinase pathway

Both inhibition and stimulation of the mitogen activated protein (MAP)-kinase pathway have been linked to the antiproliferative effects of somatostatin and its analogs. In a glioma cell line, the receptor-like PTP, PTPeta, mediated the antiproliferative effects of somatostatin through inhibition of ERK1/2[39]. Conversely, another study of sst_1-expressing CHO cells demonstrated that somatostatin robustly activated MAP-kinase, which in turn enhanced the expression of the cyclin-dependent kinase inhibitor p21, thereby inhibiting cell proliferation[40]. Another study in CHO cells demonstrated that activation of p38 MAP-kinase *via* sst_2 and sst_4 mediated the inhibitory effects of somatostatin on fibroblast growth factor induced proliferation[41].

Indirect antiproliferative mechanisms

Suppression of tumor growth may occur *via* inhibition of various circulating growth factors, including insulin-like growth factor (IGF), epidermal growth factor (EGF) and growth hormone (GH). Inhibition of GH is thought to be mediated primarily *via* sst_2 and sst_5, which are strongly expressed in the anterior pituitary[42-44]. Octreotide has been shown to suppress circulating levels of IGF-1, both *via* suppression of pituitary secretion of GH as well as through direct inhibition of IGF-1 production in the liver[45,46].

The antiangiogenic effects of octreotide have been demonstrated in multiple *in vitro* tumor models[47,48]. Octreotide has been shown to inhibit proliferating endothelial cells that over-express sst_2 and sst_5[49]. The primary mechanism of angiogenesis inhibition may be suppression of endothelial nitric oxide release[50]. Inhibition of circulating vascular-endothelial growth factor (VEGF) appears to also play a role in suppression of peritumoral vessel growth[51,52].

EARLY CLINICAL EVIDENCE FOR THE ANTIPROLIFERATIVE EFFECTS OF SSAS

Since the introduction of SSAs, multiple phase Ⅱ trials and retrospective series have demonstrated that SSA treatment is associated with prolonged survival and disease stabilization in a large proportion of patients. For example, a single-institution retrospective study of 146 patients with metastatic mid-gut NETs, 91% of whom received long term octreotide treatment, demonstrated a 5-year survival rate of 75% (compared to 19% historically)[53]. Additionally, an analysis of the US-based Surveillance, Epidemiology and End Results (SEER) database found a significant increase in survival from 1988 to 2004 compared with 1973 to 1987, coinciding with the introduction of octreotide[54].

In general, early clinical studies evaluating the dis-

ease-stabilizing effect of SSAs in patients with GEP-NETs are characterized by their lack of randomized design and enrollment of heterogeneous populations of patients with GEP-NETs. Although objective radiographic response rates have been rare (generally < 5%), the rate of tumor stabilization observed in most studies has ranged from 40%-60%, with higher rates observed in patients without documented disease progression at onset of treatment[55].

Among the first prospective studies documenting the antiproliferative effects of SSAs in GEP-NETs was one conducted by the German Sandostatin Study Group[56]. In this study, 103 patients with metastatic carcinoid and pancreatic endocrine tumors were treated with octreotide 200 μg thrice daily until evidence of radiographic progression. Among patients who had disease progression documented at treatment outset, the rate of disease stability lasting at least 3 mo was 37%, whereas among patients with documented stable disease at treatment outset, disease stability lasting at least 12 mo was documented in 54% of patients[57]. No objective tumor responses were observed. Another phase Ⅱ clinical trial testing octreotide as an antiproliferative agent in 34 patients with progressive metastatic NETs demonstrated a disease stabilization rate of 50% lasting a median of 5 mo[58].

The antiproliferative effect of intramuscular lanreotide SR 30 mg every 10 or 14 d was evaluated in a phase Ⅱ trial of 46 patients with carcinoid and pancreatic endocrine tumors. Two patients (4%) achieved an objective radiographic response while 19 patients (41%) experienced stable disease for a mean duration of 9.5 mo[59]. In another phase Ⅱ study of lanreotide SR 30 mg in 55 patients with GEP-NETs (48 with carcinoid tumors, six with gastrinomas and one with a VIPoma), 7% of 31 assessable patients achieved a partial response and 81% experienced disease stability[60]. In one study of patients with progressive tumors, participants received either octreotide LAR 30 mg or lanreotide SR 60 mg (this study considered all patients as a single cohort). Among 31 assessable patients, 14 (45%) achieved disease stability *vs* 55% who continued to progress radiographically[61]. Overall survival was considerably prolonged among patients with stable *vs* progressive disease. In multivariate analysis, pancreatic endocrine tumors appeared significantly less likely to achieve disease stabilization compared to intestinal carcinoid tumors. Extra-hepatic metastases were also associated with a poor prognosis. Table 3 summarizes the results of multiple non-randomized clinical trials evaluating the antineoplastic effects of octreotide and lanreotide in GEP-NETs.

Table 3 Summary of non-randomized clinical trials evaluating the antiproliferative effect of somatostatin analogs *n* (%)

Analog	Author	*n*	CR/PR	SD	PD
Patients with documented tumor progression					
Lanreotide	Faiss *et al*[62], 2003	22	1 (4)	7 (32)	14 (64)
Lanreotide	Aparicio *et al*[63], 2001	35	1 (3)	20 (57)	14 (40)
Octreotide	Arnold *et al*[64], 1993	52	0 (0)	19 (36)	33 (63)
Octreotide	Saltz *et al*[58], 1993	34	0 (0)	17 (50)	17 (50)
Octreotide	di Bartolomeo *et al*[19], 1996	58	2 (3)	27 (46)	29 (50)
		201	4 (1)	90 (45)	107 (53)
Patients without documented tumor progression					
Lanreotide	Wymenga *et al*[60], 1999	31	2 (6)	25 (80)	4 (13)
Lanreotide	Ducreux *et al*[59], 2000	39	2 (5)	21 (54)	16 (41)
Lanreotide	Eriksson *et al*[65], 1997	19	1 (5)	12 (63)	6 (32)
Lanreotide	Tomasetti *et al*[66], 1998	18	0 (0)	14 (77)	4 (22)
Octreotide	Tomasetti *et al*[67], 2000	16	0 (0)	14 (87)	2 (12)
Octreotide	Ricci *et al*[68], 2000	15	1 (6)	6 (40)	8 (53)
		138	6 (4)	92 (67)	40 (29)

CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease.

THE PROMID TRIAL

Although providing initial evidence for the antitumor effects of SSAs, studies described in the previous section have a number of features that prevent them from providing conclusive evidence. Examples of these features include relatively small patient cohorts, lack of a randomized placebo control group, and analysis of heterogeneous populations. As such, to prove or to disprove an antiproliferative effect of octreotide LAR 30 mg, the PROMID (Placebo-controlled, Prospective, Randomized study in patients with metastatic neuroendocrine midgut tumors) study was initiated. This randomized, double-blind, placebo-controlled, phase Ⅲ trial, was among the very few randomized trials performed in patients with this rare tumor type. To avoid confounding variables, only patients with well-differentiated inoperable or metastatic midgut tumors were included. Additionally, octreotide LAR 30 mg was the only dose of octreotide LAR evaluated.

High-level evidence of the antiproliferative effects of octreotide emerged after publication of the PROMID trial[69]. Eighty-five participants with well-differentiated carcinoid tumors originating in the distal intestine and proximal colon were randomized to receive either octreotide LAR 30 mg or placebo until radiographic evidence of progression or death. The primary endpoint was time to tumor progression. Most patients (75%) had evidence of somatostatin receptor expression as evidenced by radiotracer uptake on Octreoscan. Nearly half of patients (38%) manifested the carcinoid syndrome (flushing and/or diarrhea associated with elevation in urine 5-HIAA). Only patients with mild carcinoid syndrome who tolerated flushing without intervention or responded to treatment with loperamide and/or cholestyramine in cases of diarrhea were included.

Median time to tumor progression was 14.3 mo in the octreotide LAR 30 mg group *vs* 6.0 mo in the placebo group (P = 0.000072, Figure 3). This significantly lengthened time-to-tumor progression was seen in the overall study population, regardless of tumor functionality, chromogranin A level or age. At 6 mo, tumor progression was observed in 24% of patients on the octreotide LAR 30 mg arm *vs* 66% of patients receiving placebo (P = 0.0079). Serious adverse events were nearly evenly balanced (11 patients in the octreotide LAR 30 mg arm and 10 patients in the placebo arm). On multivariate analysis, the highest rates of disease stabilization were observed in patients

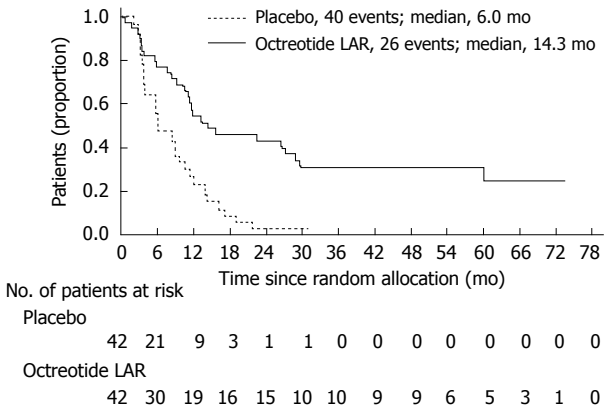


Figure 3 Kaplan Meier curve demonstrating time to tumor progression in patients treated with octreotide long-acting repeatable (LAR) *vs* placebo[69]. Log-rank test stratified by functional activity: P = 0.000072, HR = 0.34 (95% CI: 0.20-0.59).

with low hepatic tumor load (< 10%) and resected primary tumor, however both of these subgroups contained the majority of study patients. Even patients with higher hepatic tumor burden (> 10%) experienced a near doubling in time to progression on the octreotide LAR arm of the study. The small number of deaths in both treatment arms (seven in the octreotide LAR 30 mg arm; nine in the placebo arm) precluded any analysis of differences in survival.

FUTURE DIRECTIONS

Novel somatostatin analogs

NETs generally express multiple somatostatin receptors[13,70], all of which may mediate the antiproliferative effects of SSAs. These receptor subtypes can undergo heterodimerization with each other and with other receptor families (such as the dopamine receptor family), enhancing their binding affinities and internalization[71,72]. Thus, novel SSAs that bind to multiple receptor subtypes as well as analogs capable of binding to different families of receptors may prove to be effective antisecretory and antiproliferative agents in patients refractory to octreotide or lanreotide.

Pasireotide is one such novel somatostatin analog; it binds avidly to four of the five somatostatin receptors ($sst_{1,2,3}$ and sst_5). Compared with octreotide, pasireotide has a 40-, 30- and 5-fold higher binding affinity for sst_5, sst_1 and sst_3, and a slightly lower affinity for sst_2[73]. Pasireotide also has a 2-times higher binding affinity for sst_5 than endogenous somatostatin[73]. In an *in vitro* study evaluating the use of octreotide and pasireotide on HEK293 cells expressing somatostatin receptor subtype sst_2 on the cell membrane, treatment with octreotide resulted in an internalization of sst_2 receptors at 30 min whereas treatment with pasireotide did not lead to sst_2 internalization. Such findings may suggest that a persistent and more durable efficacy could be obtained with pasireotide[74].

An open-label trial evaluated the activity of pasireotide sc in patients with carcinoid syndrome whose symptoms (flushing and diarrhea) were inadequately controlled with octreotide LAR[75]. Preliminary data indicated activity in this refractory population. Future clinical trials are being designed to test the antiproliferative effects of pasireotide in neuroendocrine carcinomas. Other compounds capable of interacting with sst_2 as well as with the dopamine D2 receptor (DAD2) are in clinical development[76].

Radioactive labeling of SSAs is another promising approach to treatment of neuroendocrine malignancies which express high levels of somatostatin receptors. Early clinical trials employing ^{111}In-pentetreotide produced limited objective responses, probably due to the small particle range and short tissue penetration of Auger electrons emitted by the ^{111}In isotope[77]. The next generation of radiolabled SSAs used ^{90}Y-DOTATOC, a β-particle emitter with a tissue range of approximately 12 mm[78-80]. Objective response rates of 10%-30% were reported in phase Ⅰ and Ⅱ clinical trials. Dose-limiting side effects included bone marrow and renal toxicity.

The latest research efforts in radiolabeled SSAs have focused on ^{177}Lu octreotate, a β-and γ-emitting radionuclide with a shorter range of tissue penetration (2 mm) than ^{90}Y. A recent phase Ⅱ clinical trial reported an objective radiographic response rate of 30% among 310 patients with GEP-NETs, and a median progression-free survival duration of 40 mo[81].

CONCLUSION

SSAs were initially developed as antisecretory agents used primarily for the control of hormonal syndromes associated with NETs. In recent years, accumulating laboratory and clinical data has supported their role as antiproliferative agents, capable of stabilizing tumor growth in a large proportion of patients with metastatic carcinoid and pancreatic endocrine tumors. The recently-published PROMID study provides high-level evidence validating the role of octreotide LAR 30 mg as an antiproliferative agent in patients with metastatic carcinoid tumors of the midgut. Subset analysis suggests that patients with low tumor burden are most likely to experience disease stabilization, supporting the early use of octreotide LAR 30 mg in patients with metastatic disease. Further research efforts are underway to evaluate the use of novel SSAs, SSAs as antiproliferative agents in other types of GEP-NETs, and SSAs in combination with other anti-tumor agents.

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EDITORIAL

Gastrointestinal involvement in systemic lupus erythematosus: Insight into pathogenesis, diagnosis and treatment

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Abstract

Systemic lupus erythematosus (SLE) is an autoimmune inflammatory disease characterized by the presence of a plethora of autoantibodies and immune complex formation. Virtually every system and organ can be affected by SLE. Gastrointestinal symptoms are common in SLE patients, and more than half of them are caused by adverse reactions to medications and viral or bacterial infections. Though not as common as lupus nephritis, SLE-related gastrointestinal involvement is clinically important because most cases can be life-threatening if not treated promptly. Lupus mesenteric vasculitis is the most common cause, followed by protein-losing enteropathy, intestinal pseudo-obstruction, acute pancreatitis and other rare complications such as celiac disease, inflammatory bowel diseases, *etc*. No specific autoantibody is identified as being associated with SLE-related gastroenteropathy. Imaging studies, particularly abdominal computed tomography scans, are helpful in diagnosing some SLE-related gastroenteropathies. Most of these complications have good therapeutic responses to corticosteroids and immunosuppressive agents. Supportive measures such as bowel rest, nutritional support, antibiotics and prokinetic medications are helpful in facilitating functional recovery and improving the outcome.

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Key words: Systemic lupus erythematosus; Systemic; Vasculitis; Gastroenteropathy

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INTRODUCTION

Systemic lupus erythematosus (SLE) is a systemic autoimmune inflammatory disease with protean clinical manifestations. The gastrointestinal tract is one of the most commonly affected systems in SLE. However, most gastrointestinal manifestations are caused by adverse reactions from therapeutic agents and infections, while the symptoms related to the disease *per se* are not as common as other organ involvement such as lupus nephritis[1-6]. On the other hand, the incidence of gastrointestinal manifestations may be underestimated clinically because some of them are indistinct and may not have abdominal symptoms. One autopsy study found that 60%-70% of SLE patients had evidence of peritonitis, whereas only around 10% of them were recognized clinically[1]. It is noteworthy

that gastrointestinal vasculitis and thrombosis can lead to life-threatening ischemia, perforation and infarction, and surgical interventions are usually needed if not treated promptly with immunosuppressant. In this paper, clinically important gastrointestinal complications of SLE are reviewed for the purpose of improving treatment efficacy and outcome.

LUPUS MESENTERIC VASCULITIS

Epidemiology and pathogenesis

A number of terms have been used to describe lupus mesenteric vasculitis (LMV), including mesenteric arteritis, lupus enteritis, lupus arteritis, lupus vasculitis, gastrointestinal vasculitis, intra-abdominal vasculitis and acute gastrointestinal syndrome. LMV is one of the main causes of acute abdominal pain in SLE patients. It can be classified into an acute ischemic enteritis that involves mainly small intestine and chronic multiple ulcers occurring mainly in the colon[2]. About 8%-40% of SLE patients have acute abdominal pain during the stage of active disease[3], but LMV is generally an uncommon condition in SLE patients. In Asia, the reported overall prevalence of LMV in patients with SLE is 2.2%-9.7%[4-6]. However, the prevalence of LMV in America seems to be much lower: 0.9%[7]. Ju *et al*[8] reported that the global prevalence of LMV ranges from 0.2% to 9.7% among all SLE patients and from 29% to 65% in patients who had acute abdominal pain. LMV occurs almost always in patients with active disease[6].

The predisposing factors for LMV are unknown. The proposed trigger factors include bacterial infections that lead to changes of intestinal flora, cytomegalovirus infection, eosinophilia, non steroidal anti-inflammatory drugs, chemicals, metallic particulates, animal viruses, helminth infection, caffeine, phosphodiesterase-4-inhibitors, adenosine diphosphate, certain foods and herbal medicines[6].

Inflammatory vasculitis secondary to immune complex deposition and thrombosis of the intestinal vessels secondary to circulating anti-phospholipid antibodies are the proposed pathogenic mechanisms of LMV[9-12]. Both types of microvasculopathy can activate each other reciprocally, resulting in worsening cascades of vasculitis and thrombosis. Autoantibodies, such as lupus anticoagulant, anti-cardiolipin antibody and anti-β2-glycoprotein antibody, are associated with LMV. A study suggests that some cryptic antigens which can stimulate the production of anti-endothelial antibodies are exposed when endothelial cells are disrupted[10]. In a study by Kwok *et al*[6], the authors found that the serum levels of anti-endothelial cell IgG were significantly higher in SLE patients with LMV than in those without LMV or in healthy controls. Macroscopically, the appearance of LMV varies from segmental edema to ulceration, gangrene and perforation[8]. Both small arteritis and venulitis are found in LMV. Microscopically, fibrinoid necrosis of subserosal vessels and leukocytoclasis on the vascular wall, as well as edematous submucosa with mild diffuse inflammatory infiltration of mononuclear cells, can be observed. In the muscular layer, intravascular fibrin thrombus and hemorrhage around the small veins can be found.

Clinical features

Both the inflammatory and thrombotic mesenteric vasculopathy of LMV can cause mesenteric ischemia. LMV can cause very severe abdominal symptoms and signs and sometimes is diagnosed as acute surgical abdomen. Typically, the abdominal pain caused by LMV is diffuse in pattern, in some cases accompanied by rebound tenderness and abdominal muscle guarding. The symptoms of LMV vary from mild, nonspecific abdominal pain, bloating or loose stool, to necrosis and intestinal perforation which manifest as severe extensive gastrointestinal bleeding or acute surgical abdomen. It is noteworthy that in LMV patients with bowel perforation, the typical signs can be absent. Other manifestations of LMV include anorexia, nausea, vomiting, dysphagia, hematemesis, postprandial fullness, diarrhea and melena. Kishimoto *et al*[13] reported two cases of "acute gastrointestinal distress syndrome". Patients with this syndrome have recurrent lupus enteritis on the basis of mesenteric vasculopathy characterized by reversible intestinal wall edema accompanied by significant hypocomplementemia. These attacks are not complicated by bowel infarction, perforation or hematachezia and respond quickly to corticosteroid treatment.

Diagnosis

Accurate diagnosis of LMV is critical to allow prompt treatment to avoid unnecessary surgical intervention. As the clinical symptoms and laboratory parameters are non-specific, and bowel specimens are not always available, the diagnosis of LMV relies on abdominal computed tomography (CT) scan which allows both the bowel wall and the abdominal vasculature to be visualized. Advances in CT technology have been extremely helpful in detecting ischemia and for evaluating the causes of abdominal pain.

Common CT findings in patients with LMV include dilated bowel, focal or diffuse bowel wall thickening, abnormal bowel wall enhancement which is also called "target sign", mesenteric edema, stenosis or engorgement of mesenteric vessels which is also called "the comb sign" and ascites (Figure 1). Segmental or multifocal involvement of the small and large bowel loops with intervening normal bowel segments indicates ischemic change, which is almost always indicative of vasculitis.

Ultrasonography is also useful for both the diagnosis and follow-up of LMV. Small intestinal wall edema and thickening can be visualized under ultrasonography. Irregular thickening and projection of folds in multiple segments of the duodenum and the terminal ileum accompanied by "thumb print" signs in double-contrast radiography suggest ischemic changes. Gastroscopy and colonoscopy can show ischemia and ulcerative changes. However, endoscopy-guided biopsy might not yield a definitive diagnosis of LMV because the affected vessels are usually located in an inaccessible area. Laparoscopy can be used in the diagnosis of LMV[8,9].



Figure 1 Stenosis of artery mesenterica superior (SMA) in a patient with systemic lupus erythematosus.

Management

Early diagnosis and appropriate intervention can avoid potentially fatal complications of LMV. Because the primary lesions of LMV are inflammatory ischemic vasculitis, immediate and aggressive anti-inflammatory immunosuppressive treatment should be initiated as soon as the diagnosis of LMV is made. The treatments include high dose intravenous infusion of methylprednisolone or an equivalent agent and complete bowel rest. For patients with recurrent LMV and those who do not have adequate response to intravenous prednisolone alone, intravenous cyclophosphamide should be initiated. The initial dosage of cyclophosphamide is 1 mg/kg daily and the dosage is gradually tapered when LMV is stabilized[6]. In Kim's study, they found that a bowel wall thickness greater than 9 mm usually indicated the presence of recurrent LMV and should be considered a high risk factor for recurrence[14]. They suggested that for patients with high risk of recurrence, immunosuppressive agents should be initiated as early as possible.

When a rapid response to immunosuppressive therapy is not achieved, surgical intervention for possible bowel perforation or large area of ischemia should be considered. Early laparotomy within 24 to 48 h is critical for improving the prognosis of LMV patients. Medina *et al*[15] found that 10 of 11 LMV patients who underwent surgery after 48 h died, while none of 33 patients who were operated on within 24-48 h died.

Prognosis

The prognosis of LMV varies in reports from different areas of the world. This might be due to genetic differences. Reports from Europe and North America indicate that the prognosis of LMV is poor and some fatalities can occur. Some reports claimed that the mortality of LMV could be as high as 50%[6,15]. The prognosis of LMV depends on the extent of vascular involvement, the prompt implementation of immunosuppressive therapy, and the time of surgical intervention. On the basis of published reports, the prognosis of LMV can be improved if abdominal CT is used to aid the diagnosis and if prompt immunosuppressive therapy is implemented.

PROTEIN-LOSING GASTROENTEROPATHY

Epidemiology and pathogenesis

Protein-losing gastroenteropathy (PLGE) is a condition characterized by profound edema and severe hypoalbuminemia secondary to excessive loss of serum protein from the gastrointestinal tract, clinically indistinguishable from nephrotic syndrome. Clinically significant PLGE seems uncommon in SLE. Besides two large series reported by Mok and our group with 16 and 15 patients respectively, most are isolated case reports or small series[16,17]. So far, less than 60 patients with SLE-related PLGE have been reported in the literature, and the majority of these patients are Asians; whether this is due to genetics or environmental factors remains to be elucidated. In Mok's study, the point prevalence of their Chinese patients is 3.2% for PLGE, while in our series the prevalence is 1.9%. PLGE may occur in SLE patients of any age and occurs with a female predominance. In most circumstances, the typical features of PLGE develop before the diagnosis of SLE. In our study, 53.3% of patients had PLGE as the initial presentation of SLE, but PLGE could also occur 17 years after the establishment of SLE[17]. Gornisiewicz *et al*[18] have concluded that PLGE often happens in patients with clinically severe SLE with multiple system involvement.

The exact pathogenesis of SLE-related PLGE is still unclear. Mucosal ulceration, non-necrotizing mesenteric or intestinal blood vessel vasculitis, increase in capillary permeability caused by intravascular activation and conversion of complement, cytokine- (such as tumor necrosis factor-α and interleukin-6) or complement-mediated vascular or mucosal damage, and intestinal lymphangiectasia have been postulated as the pathogenic mechanisms[16,19].

Clinical features

The most predominant clinical manifestation of PLGE is profound peripheral pitting edema. Patients may have pleural, pericardial effusion and ascites due to severe hypoalbuminemia. Nausea, vomiting and diarrhea are also common in these patients. Diarrhea is present in 50% of cases, which is generally liquid in nature and can be as frequent as 20 times a day, but steatorrhea is absent[17,20]. No particular autoantibodies have been found to be associated with PLGE. Severe hypoalbuminemia and hypocomplementemia are the most predominant laboratory findings of PLGE. Kim *et al*[19] found in their study that hypercholesterolemia was common in SLE-related PLGE, but rare in idiopathic PLGE[16]. This might be due to the leakage of cholesterol-rich lipoprotein particles in the intestinal lymph. Most PLGE patients also have hypoglobulinemia because immunoglobulin can also leak into the intestinal lumen. The presence of anti-dsDNA and anti-ENA antibodies is not significantly different from patients without PLGE. Eighty percent of PLGE in our series occurred at the stage of active lupus, and

non-specific chronic inflammation were detected in all pathological specimens obtained.

Diagnosis

The diagnosis of SLE-related PLGE mainly relies on the exclusion of other causes of hypoalbuminemia such as lupus nephritis, abnormal liver function, decreased protein synthesis or malabsorption. In recent years, Tc-99m albumin scintigraphy has become the most frequently used diagnostic method. It is noninvasive and safe in demonstrating gastrointestinal loss of blood albumin to intestinal lumen. It also has the potential to localize protein leakage and can be used to monitor the efficacy of treatment. Gastroenteroscopy is usually not diagnostic, as 50% of SLE-related PLGE patients present with non-specific intestinal wall edema and 10% of patients have no abnormalities under endoscopy examination[21]. Histologically, lymphangiectesia, edematous villi and non-specific inflammation can be found in the intestine, but histology can also be normal because of the inaccessibility of the involved area under gastroendoscopy. An elevated α-1 antitrypsin can be used as a diagnostic alternative[22].

Management

Due to limited number of patients, there is no controlled clinical trial to demonstrate the efficacy of treatment for SLE-related PLGE. Corticosteroid is the mainstay of treatment. Response to corticosteroid alone is excellent in more than 60% of patients. For those refractory to steroid, immunosuppressive agents such as azathioprine, cyclosporine A and cyclophosphamide could be added into the therapeutic regimen. Albumin infusion, nutritional support and diuretics are used as supplemental measures for the treatment. Octreotide can reduce intestinal microvasculature blood flow, decrease local lymph formation and ameliorate lymphatic dilatation, therefore can also be used in the treatment of PLGE. In addition, octreotide has immunomodulatory effects since it specifically binds with the somatostatin receptor. Prophylaxis for thromboembolic complications with warfarin should be considered in patients with severe and persistent protein loss, especially if antiphospholipid antibodies are present[16-18,20].

Prognosis

The outcome of most SLE-related PLGE cases is generally good since most patients respond well to steroid therapy. Relapse of PLGE occurs in 20%-30% of patients, particularly in patients undergoing maintenance therapy with low-dose prednisolone alone, but they respond again to an increased dosage of steroid. Mok *et al*[16] suggested that long-term maintenance treatment with low dose prednisolone plus azathioprine could reduce the rate of recurrence.

INTESTINAL PSEUDO-OBSTRUCTION

Prevalence and pathogenesis

SLE-related intestinal pseudo-obstruction (IPO) is a rare but well-recognized clinical syndrome that reflects the dysfunction of the visceral smooth muscle, the enteric nerve and/or the visceral automatic nervous system[23]. It usually coincides with ureterohydronephrosis and/or interstitial cystitis, rarely with biliary dilatation (megacholedochus)[24]. IPO is associated with ureterohydronephrosis in 63.3% of cases[25]. IPO may appear during the course of SLE, but it can also be the initial presentation of SLE[26]. It usually occurs in patients with active lupus. So far, only 28 cases have been reported in the English literature; half of the cases were Oriental and female patients predominated[23].

The pathogenesis of SLE-related IPO is unknown. Histopathological evidence of intestinal leiomyocyte damage suggests a systemic autoimmune process targeting smooth muscle cells[24]. Vasculitis leading to chronic ischemia of the bowel smooth muscle, which in turn leads to muscular damage and hypomotility, has been postulated as one of the possible mechanisms. Another possible mechanism is an intrinsic muscle dysmotility affecting the muscularis propria. The apparently high association between pseudo-obstruction and ureterohydronephrosis suggests a possible common smooth muscle dysmotility due to primary myopathy or neurogenic pathology, secondary to either immune complex-mediated vasculitis or common circulating autoantibodies against smooth muscle. Biopsied specimens have shown fibrotic process and atrophy in the muscularis layer, decreased number of smooth muscle cells and inflammatory cell infiltration associated with fibrinoid deposits, indicative of vasculitis[26].

Clinical features

The characteristic clinical manifestation of IPO is ineffective intestinal propulsion with the presence of clinical features of intestinal obstruction without an identifiable organic obstructive lesion and an abdominal distension with a very sluggish or absent peristalsis. Antroduodenal manometry demonstrates intestinal hypomotility and esophageal aperistalsis. Symptoms of IPO include a subacute onset of abdominal pain, nausea and vomiting, abdominal distension, constipation, diarrhea and weight loss. Laboratory findings are non-specific. Anti-proliferative cell nuclear antigen antibody (anti-PCNA) was found to be more frequently detected than in lupus patients without IPO in one study[27]. Mok *et al*[28] reported that SLE-related IPO patients had higher frequency of positive anti-Ro and anti-RNP antibodies compared to lupus patients without IPO.

Radiological examination can detect dilated fluid-filled bowel loops, with thickened bowel wall and multiple fluid levels. Bilateral ureter dilatation with a reduced urinary bladder capacity can be found if ureterhydronephrosis is the concurrent situation. Abdominal CT scan frequently reveals the presence of dilated small and large bowel with thickened intestinal wall.

Pathological examination of the gastrointestinal tract of SLE-related IPO patients can reveal widespread myocyte necrosis in the muscularis propria with active inflammatory cell infiltration, severe atrophy of muscularis, active serositis with serosal thickening and fibrosis, but little or no evidence of vasculitis and absence of thromboembolism.

Diagnosis

The diagnosis of this clinical syndrome is based on imaging findings consistent with the presence of dilated small and large bowel loops with thickened intestinal wall and multiple fluid levels. Other possible causes of intestinal obstruction should be excluded. Invasive diagnostic procedures should be avoided.

Management

Corticosteroids, immunosuppressive agents combined with supportive measures such as parenteral nutrition, oral broad-spectrum antibiotics to diminish bacteria overgrowth and pharmacological stimulation of small bowel motility are effective medical management options for SLE-related IPO. Timely diagnosis and early intervention are critical to rehabilitate the peristaltic activity of both the gastrointestinal tract and the genitourinary viscera; delayed treatment has been associated with failure to regain functional peristalsis and leads to histopathological progression to fibrosis and atrophy of the intestinal wall, and secondary impairment of the myenteric plexuses[24]. SLE-related IPO usually responds well to high dose corticosteroids. Erythromycin in particular is the appropriate antibiotic in this situation due to its prokinetic effect. Other prokinetic agents such as cisapride and octreotide are also helpful in stimulating small bowel motility. Octreotide appears to be effective in improving both clinical symptoms and manometric patterns[26]. Azathioprine, cyclophosphamide and cyclosporine A are the immunosuppressive agents that could be used as one component of a maintenance therapeutic regimen with oral corticosteroids. Other immunosuppressive agents against B cells, immune complex formation and pathogenic autoantibody formation could be the alternatives.

Prognosis

High dose intravenous corticosteroid treatment is effective in most patients, leading to clinical remission and disappearance of abnormal imaging findings. Long-term outcome of SLE-related IPO varies. Some patients may have recurrent attacks of IPO without other major organ involvement despite maintenance treatment with steroids and immunosuppressive agents. The reported mortality rate is 18%[26]. Early diagnosis and prompt treatment is critical to improve the overall outcome of SLE-related IPO patients.

PANCREATITIS

Epidemiology and pathogenesis

About 160 cases of SLE-related acute pancreatitis have been reported in the literature. Pancreatitis is a rare but life-threatening complication of SLE and our knowledge about this complication is mainly based on individual case reports. Based on the literature reports, the annual incidence of SLE-related pancreatitis is estimated to be 0.4-1.1/1000 lupus patients[29,30]. Reports from Europe and the United States showed that the rate of pancreatitis with SLE is between 0.7%-4%[31,32]. However, the rate of SLE-related pancreatitis may be underestimated because cases of subclinical pancreatitis with elevated pancreatic enzymes but no symptoms are not diagnosed or reported. It is estimated that 30.5% of asymptomatic SLE patients have hyperamylasemia[33].

Sixty percent of cases develop acute pancreatitis within 2 years of the diagnosis of lupus, and in 22% of patients, pancreatitis is the initial clinical presentation. In most cases, acute pancreatitis is associated with active lupus. In addition to traditional predisposing factors such as hypertriglyceridemia, steroid and azathioprine use were proposed as the possible causes of pancreatitis in lupus patients. However, in Pascual-Ramos's study, they could not prove the association between steroid and/or azathioprine administration and the development of pancreatitis even after they "rechallenged" the onset of pancreatitis with these medications[34].

The pathogenic mechanism of SLE-related pancreatitis is unclear. Vascular damage has been stressed as a cause of this problem. Necrotizing vasculitis, occlusion of arteries and arterioles by thrombi resulting from severe hypertension or antiphospholipid syndrome, intimal thickening and proliferation and immune complex deposition with complement activation in the wall of pancreatic arteries have been postulated[31]. Sixteen specimens of pancreatic tissue from SLE-related pancreatitis patients were examined in one series; evidence of inflammation and necrosis could be found in all specimens, but vasculitis could only be found in one case[31]. It is possible that an autoimmune reaction involving abnormal cellular immune response or antibody reaction, rather than vasculitis, is responsible for the inflammation reaction.

Clinical features

Eighty-eight percent of SLE-related pancreatitis cases have abdominal pain; in only 23% of them the pain radiates to the back. Two-thirds of patients have nausea and vomiting, and half of them have fever. Diarrhea is uncommon and a few patients have panniculitis.

Elevated serum amylase and lipase are the most commonly detected biochemical abnormalities. Additional biochemical abnormalities include hypoalbuminemia, abnormal liver function tests, elevated serum creatinine and hypocalcemia. Anti-La antibody is the only autoantibody reported to be associated with this particular complication[32].

Diagnosis

The diagnosis is based on the laboratory evidence of elevated serum amylase or lipase levels. Clinical symptoms and suggestive tomographic findings are helpful. However, lupus patients may develop acute pancreatitis secondary to other "non-SLE" causes such as mechanical (including cholelithiasis), toxic chemicals (such as alcohol ingestion, some medications), hypertriglycemia and hypocalcemia, as well as viral infections or sepsis. The diagnosis of SLE-related pancreatitis can only be confirmed when these possible causes are excluded.

Management

Corticosteroids should be used as the medical management of SLE-related acute pancreatitis as long as this drug can be excluded as the cause of pancreatitis. Immunosuppressive agents such as azathioprine or cyclophosphamide can be used in combination with corticosteroid. In severe cases, plasmapheresis and intravenous gamma-globulin infusion may be helpful.

Prognosis

As many as 57% of SLE-related acute pancreatitis cases may develop complications if not treated promptly[30]. Many of these complications can be fatal, with a mortality rate of 45%, whereas it is only 3% in patients without complications. Lupus activity is significantly associated with increased mortality. It is reported that SLE-related acute pancreatitis concurrent with central nervous system and cardiac involvement has the highest mortality rate. Increased serum creatinine, hypoalbuminemia, anti-DNA antibodies, thrombocytopenia, low complement, hypocalcemia, hyperglycemia and elevated liver enzymes are risk factors for increased mortality.

About 22% of patients may experience recurrent acute pancreatitis attacks, while 12% of patients develop pancreatic pseudocysts and 5%-14% become chronic[30,32].

OTHER SLE-RELATED GASTROINTESTINAL PROBLEMS

Celiac disease

The coexistence of SLE and celiac disease is rare. So far, only 17 cases have been reported in the literature. Both diseases have an autoimmune nature and share HLA-B8 and HLA-DR3 histocompatibility antigens[35]. Celiac disease can occur before or after the diagnosis of lupus[36]. Most patients have positive serum antigliadin antibodies and histological findings of duodenal biopsy which are consonant with celiac disease. Patient's response to steroid along with gluten-free diet is excellent. The prognosis is generally good.

Inflammatory bowel disease

The coexistence of SLE and inflammatory bowel disease (IBD) is difficult to diagnose since both diseases share some common gastrointestinal features and some medications used in treating IBD may cause drug-induced lupus. The estimated prevalence of ulcerative colitis (UC) in SLE patients is around 0.4%[37]. So far, 27 cases of SLE-related UC have been reported, and numbers of SLE-related Crohn's disease are even less. UC may occur either before or after the diagnosis of SLE. The majority of patients have excellent response to steroids combined with hydroxychloroquine or azathioprine. However, some patients with Crohn's disease may have life-threatening massive gastrointestinal bleeding and need high dosage methylprednisolone[38]. The prognosis of SLE-related IBD is usually good.

Eosinophilic enteritis

Eosinophilic enteritis is a rare clinical condition. SLE-related eosinophilic enteritis is even rarer. Only 3 cases of SLE-related eosinophilic enteritis have been reported in the literature[39]. The clinical symptoms include abdominal pain, nausea, vomiting and sometimes diarrhea. Peripheral hypereosinophilia presents in most of the patients. The diagnosis depends on clinical symptoms and intestinal biopsy which shows eosinophils in the deep layers of the intestinal wall[39]. The recommended treatment regimen is prednisone 0.5-1 mg/kg per day in divided doses for 7-10 d followed by slow tapering over 2 to 3 mo. Immunosuppressive agents can be used in patients with recurrence or corticosteroid non-responders.

Pneumatosis cystoides intestinalis

This is an uncommon disorder characterized by the presence of gas within the walls of the gastrointestinal tract. The most common rheumatic disease that is associated with pneumatosis cystoides intestinalis (PCI) is systemic sclerosis. Only 14 SLE-related PCI cases have been reported in the literature. It might be caused by injury of the mucosa and immune barrier due to lupus vasculitis and impaired healing due to corticosteroid therapy[40]. Pathological evidence of vasculitis can be found in at least half of the patients. Increased intraluminal pressure, mucosal injury and production of gas from bacteria in the mucosa may be involved in the pathogenesis of SLE-related PCI. The diagnosis depends on the abdomen findings on CT, which show thickened bowel walls with multiple linear and cystic radiolucencies. Therapeutic strategy for each PCI patient should be individualized. Oxygen inhalation or hyperbaric oxygen therapy is reported to promote removal of the gas from the cysts. Antibiotics are effective in reducing bacterial overgrowth and gas production. Bowel rest and prokinetic agents are the supportive measures in treating patients with SLE-related PCI.

CONCLUSION

Gastrointestinal manifestations are common in SLE patients, but most of them are due to adverse reactions to medications and infection. SLE-related gastrointestinal involvement is not rare, and sometimes can be life-threatening. Most SLE-related gastrointestinal complications are caused by vasculitis and immune complex deposition, and respond well to corticosteroids and immunosuppressive agents. Early diagnosis and timely treatment are critical to improve the prognosis. Supportive measures such as bowel rest are beneficial, and in some situations such as IPO and PCI antibiotics are helpful to facilitate functional restoration.

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TOPIC HIGHLIGHT

Marcela Kopacova, Associate Professor, MD, PhD, *Series Editor*

Small intestinal bacterial overgrowth syndrome

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Abstract

Human intestinal microbiota create a complex polymicrobial ecology. This is characterised by its high population density, wide diversity and complexity of interaction. Any dysbalance of this complex intestinal microbiome, both qualitative and quantitative, might have serious health consequence for a macro-organism, including small intestinal bacterial overgrowth syndrome (SIBO). SIBO is defined as an increase in the number and/or alteration in the type of bacteria in the upper gastrointestinal tract. There are several endogenous defence mechanisms for preventing bacterial overgrowth: gastric acid secretion, intestinal motility, intact ileo-caecal valve, immunoglobulins within intestinal secretion and bacteriostatic properties of pancreatic and biliary secretion. Aetiology of SIBO is usually complex, associated with disorders of protective antibacterial mechanisms (e.g. achlorhydria, pancreatic exocrine insufficiency, immunodeficiency syndromes), anatomical abnormalities (e.g. small intestinal obstruction, diverticula, fistulae, surgical blind loop, previous ileo-caecal resections) and/or motility disorders (e.g. scleroderma, autonomic neuropathy in diabetes mellitus, post-radiation enteropathy, small intestinal pseudo-obstruction). In some patients more than one factor may be involved. Symptoms related to SIBO are bloating, diarrhoea, malabsorption, weight loss and malnutrition. The gold standard for diagnosing SIBO is still microbial investigation of jejunal aspirates. Non-invasive hydrogen and methane breath tests are most commonly used for the diagnosis of SIBO using glucose or lactulose. Therapy for SIBO must be complex, addressing all causes, symptoms and complications, and fully individualised. It should include treatment of the underlying disease, nutritional support and cyclical gastro-intestinal selective antibiotics. Prognosis is usually serious, determined mostly by the underlying disease that led to SIBO.

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Key words: Bacterial overgrowth; Breath test; Hydrogen; Methane; Small intestine

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INTRODUCTION

Human intestinal microbiota create a complex polymicrobial ecology. This is characterised by its high population density, wide diversity and complexity of interaction. The duodenum and proximal jejunum normally contain small numbers of bacteria, usually lactobacilli and enterococci, gram-positive aerobes or facultative anaerobes ($< 10^4$ organisms per mL). Coliforms may be transiently present ($< 10^3$ bacteria per mL) and anaerobic *Bacteroides* are not found in the jejunum in healthy people. Up to one third of jejunal aspirates might be sterile in healthy volunteers. The distal ileum is a transition zone between sparse populations of aerobic bacteria of the proximal small intestine and very dense populations of anaerobic micro-organisms in the large bowel[1-3]. The epithelial surface of the small intestine in a healthy human is not colonised. Occasional groups of bacteria can be found in low concentrations within the lumen. Bacteria do not form clusters and spatial structures, and the luminal contents are separated from the mucosa by a mucus layer[4].

Any dysbalance of this complex intestinal microbiome, both qualitative and quantitative, might have serious health consequences for a macro-organism, including small intestinal bacterial overgrowth syndrome (SIBO).

DEFINITION

SIBO is a very heterogeneous syndrome characterised by an increased number and/or abnormal type of bacteria in the small bowel. Most authors consider diagnostic of SIBO to be the finding of $\geqslant 10^5$ bacteria [i.e. colony-forming units (CFU)] per mL of proximal jejunal aspiration. The normal value is $\leqslant 10^4$ CFU/mL[3, 5-7].

PREVALENCE

The overall prevalence of SIBO in the general public is unknown. In general, SIBO is substantially underdiagnosed. There are several reasons for this fact. Some patients may not seek healthcare or SIBO may not be properly diagnosed by medical investigations. SIBO might be asymptomatic or with non-specific symptoms only, and last but not least, all symptoms might be incorrectly ascribed to the underlying disease (leading to SIBO). Of course, diagnostic yield also depends on the methods used for investigation. According to different studies with the investigation of small sets of clinically healthy people as a control, findings consistent with SIBO were found in 2.5% to 22%[8-17].

In particular diseases and disorders, literature data on prevalence differ substantially. For instance, the prevalence of SIBO in patients fulfilling diagnostic criteria for irritable bowel syndrome was 30%-85%[9-11,16,18,19]. The prevalence of SIBO in coeliac disease non-responding to a gluten-free diet was up to 50%[20]. In liver cirrhosis, SIBO was diagnosed in more than 50% of cases[21,22]. In a small group of elderly people (70 to 94 years old) with lactose malabsorption, SIBO was documented in 90%[23]. An interesting study was performed on asymptomatic morbidly obese subjects and SIBO was found in 17% (compared to 2.5% in non-obese persons)[15].

AETIOLOGY

There are several endogenous defence mechanisms for preventing bacterial overgrowth: gastric acid secretion, intestinal motility, intact ileo-caecal valve, immunoglobulins within intestinal secretion and bacteriostatic properties of pancreatic and biliary secretion[24].

The aetiology of SIBO is usually complex, associated with disorders of protective antibacterial mechanisms (e.g. achlorhydria, pancreatic exocrine insufficiency, immunodeficiency syndromes), anatomical abnormalities (e.g. small intestinal obstruction, diverticula, fistulae, surgical blind loop, previous ileo-caecal resections) and/or motility disorders (e.g. scleroderma, autonomic neuropathy in diabetes mellitus, post-radiation enteropathy, small intestinal pseudo-obstruction). In some patients more than one factor may be involved. "Aetiological" and "predisposing" factors cannot be separated in some patients. SIBO may occur in elderly people without any evident underlying small intestinal pathology.

In some cases, a vicious circle arises: an underlying disease is complicated by SIBO and then SIBO directly (as a morphological impact) or vicariously (by malabsorption or nutrient deficiency) causes further deterioration of the underlying disease.

Out of all diseases and disorders associated with SIBO (listed below in detail), 90% of cases comprise small intestinal motility disorders (of various aetiology) and chronic pancreatitis[2].

Achlorhydria

Achlorhydria (due to chronic atrophic gastritis) and long-term administration of proton pump inhibitors may cause bacterial overgrowth in the stomach and duodenum. Proton pump inhibitors not only increase duodenal bacterial colonisation but also accelerate intestinal transit[24].

Exocrine pancreatic insufficiency

Chronic pancreatitis is complicated by SIBO in 30%-40% of cases[7,25]. Multiple factors can be involved: exocrine pancreatic insufficiency (with absence of anti-bacterial effect of proteolytic enzymes), abnormal chyme in the small intestinal lumen, motility disorders, administration of painkillers and ongoing alcohol consumption in some of patients. Cystic fibrosis is also associated with increased risk of SIBO. Fridge *et al*[26] diagnosed SIBO in 14/25 (56%) patients with cystic fibrosis. SIBO may be a causative fac-

tor of diarrhoea in advanced pancreatic cancer[27] apart from pancreatic exocrine insufficiency, chemotherapy or previous surgery.

Immunodeficiency syndromes

Various immunodeficiency syndromes, such as IgA deficiency, common variable immunodeficiency, AIDS and others, are complicated by miscellaneous infection complications, including SIBO[28,29].

Small intestinal obstruction and stagnation

All anatomical pathology associated with small intestinal obstruction and stagnation could be associated with SIBO, e.g. strictures, adhesions, tumours of the small bowel. Large and/or multiple duodenal and jejunal diverticula are often complicated by SIBO. Sequelae of previous abdominal surgery (afferent loop syndrome after Billroth-Ⅱ gastric resection, Roux-en-Y stasis syndrome, bariatric bypass surgery) may also lead to SIBO (with metabolic and nutritional disarrangement)[7,30-32]. Small intestinal pseudo-obstruction and some neurological diseases (e.g. myotonic dystrophy, Parkinson disease, Chagasic enteropathy) can be complicated by SIBO that is responsible for malabsorption and weight loss[33-36]. Spinucci *et al*[37] described an interesting case of endogenous ethanol production in a patient with chronic intestinal pseudo-obstruction and SIBO.

Tursi *et al*[38] investigated bacterial overgrowth in the small bowel in patients with acute diverticulitis of the colon. Small intestinal overgrowth was found in 53/90 (59%) subjects. The authors assumed that the primary mechanism is a slow large bowel transit with stasis of faeces in the colon. This results in dysmicrobia in the large bowel with metabolic changes and induction of inflammation. Subsequent reverse peristalsis facilitates colonisation of the small intestine by bacteria coming from the large bowel. SIBO deteriorates symptoms of acute colonic diverticulitis, protracts the course of the disease and thus could be an independent risk factor for future relapses of acute diverticulitis of the large bowel. Rifaximin was effective in the treatment of both acute colonic diverticulitis and SIBO in these patients[38].

Irritable bowel syndrome

The aetio-pathogenesis of irritable bowel syndrome has not yet been satisfactorily clarified. Symptoms of SIBO and irritable bowel syndrome overlap to a large degree. As mentioned earlier, SIBO is frequently found in persons fulfilling criteria of irritable bowel syndrome (30%-85%)[9-11,16,18,19]. According to authors of the bacterial hypothesis, SIBO is the primary event and irritable bowel is secondary to SIBO. In some patients, the onset of irritable bowel is preceded by infective gastroenteritis (so-called post-dysenteric bowel disturbance)[39]. Analysis of the microbial genome found different faecal microbiota in healthy people and patients with irritable bowel (e.g. phylotypes *Coprococcus*, *Collinsella*, *Coprobacillus*)[40-42]. Believers in an opposite hypothesis stated that irritable bowel is a primary factor (with motor disturbance, visceral afferent hypersensitivity, psycho-social dysfunction) in which motility disorders enable "secondary" bacterial overgrowth[40,43]. A third group of authors recommend strict distinction between irritable bowel syndrome (the hydrogen breath test with lactulose must be negative) and SIBO (in such a case, it is not irritable bowel despite the diagnostic criteria having been met) in patients with identical symptoms[44]. The last authors stated an opinion that SIBO does not play any significant role in the pathogenesis of irritable bowel[45].

Pimentel *et al*[12] found abnormal lactulose breath test results in 93/111 (84%) patients with irritable bowel syndrome. Successful treatment of SIBO using neomycin (in 35% of patients) was associated with relief of subjective symptoms. There was another interesting finding in this study: a subgroup of persons with a methanogenic phenotype was associated with constipation in 100% (constipation-predominant irritable bowel syndrome)[12]. Another study found that methanogenic status was never associated in irritable bowel syndrome with diarrhoea and Crohn's disease or ulcerative colitis with diarrhoea[13]. The association of methanogenic phenotype and constipation was also revealed by other authors[8].

Coeliac disease

A wide range of 9% to 55% of patients have been diagnosed with SIBO as a complication of coeliac disease[20,46-48]. The prevalence of SIBO is high, especially in patients who do not respond to a gluten-free diet and/or have lactose intolerance[20,46,47].

Crohn's disease

SIBO is frequently found in Crohn's disease (in about 25%). Loss of the ileo-caecal valve (due to previous ileocaecal resection) and/or large entero-enteric and enterocolic fistulae are important predisposing factors[49-55]. Castiglione *et al*[56] found bacterial overgrowth more frequently in those who underwent surgery (30%) compared to non-operated patients (18%). Furthermore, SIBO may mimic an acute flare of Crohn's disease (including increased bowel movements and lower body weight)[57]. Smokers may exhibit increased H_2 production which could lead to false positive test results. However, in the study by Klaus *et al*[57] there was no difference in the proportion of smokers and their respective daily consumption of cigarettes between patients with Crohn's disease with and without SIBO.

Short bowel syndrome

The problem of short bowel syndrome is not limited only to the reduced absorptive surface area. The loss of the ileocaecal valve and the loss of the ileal break from resection of the distal small bowel would accelerate the transit of chyme throughout the entire gastrointestinal tract. Undigested food becomes a substrate for bacterial fermentation. Large intestinal bacterial flora colonise proximally into the small intestine to result in SIBO. Because digestion and absorption cannot be completed without adequate time, these patients face chronic postprandial diarrhoea.

These problems may be exacerbated by SIBO that further accelerates transit and worsens digestion, absorption and malnutrition[58].

SIBO is an independent negative factor deteriorating adaptation of the small intestine in children after excessive bowel resections. SIBO lengthens the dependence of these patients on total parenteral nutrition and deteriorates malabsorption and hepatopathy associated with short bowel syndrome[59,60]. SIBO may lead to intestinal failure in these patients[61].

Non-alcoholic steatohepatitis

Wigg *et al*[17] found a higher prevalence of SIBO (11/22, 50%) in non-alcoholic steatohepatitis (NASH) compared to healthy control subjects (5/23, 22%). Higher values for the xylose-lactulose test in patients with NASH correlated with higher serum levels of tumor necrosis factor-α (TNF-α). However, they were not associated with increased intestinal permeability or increased serum endotoxin[17]. In another study of NASH[62], SIBO was diagnosed in half of the patients (6/12) but only in one subject (1/11, 9%) in the healthy control group. Treatment with ciprofloxacin suppressed bacterial overgrowth, increased serum insulin and decreased endogenous ethanol production but did not influence serum acetylated ghrelin (half values compared to controls). Changes in fasting insulin and ethanol following ciprofloxacin suggest that these parameters may be influenced by small intestinal bacterial activity[62]. In an experimental model of NASH in rats, there was a slower transit time and higher quantity of coliform bacteria (*Escherichia coli*). Treatment with gentamicin (cidomycin) accelerated the transit time, decreased TNF-α levels and alleviated severity of liver involvement in experimental animals. Thus SIBO might play an important role in the pathogenesis of NASH[63].

Liver cirrhosis

Portal hypertension in liver cirrhosis substantially changes the intraluminal milieu of the gut. Liver cirrhosis is an independent risk factor for SIBO. Small intestinal motility disorder, especially slow transit in advanced liver disease (Child-Pugh C) may partake in SIBO[64,65]. SIBO was diagnosed in 50%-60% of patients with liver cirrhosis[66,67]. SIBO is a risk factor for the development of spontaneous bacterial peritonitis[22,68], however, its role in the pathogenesis has not yet been fully clarified[22]. Prevalence of SIBO was higher in those patients with liver cirrhosis who had spontaneous bacterial peritonitis (14/20, 70%) compared to those without it (4/20, 20%)[64]. However, this finding was not confirmed in other studies[66]. SIBO might correlate with systemic endotoxaemia[69]. It is necessary to remind ourselves that glucose hydrogen breath test in liver cirrhosis correlates only to a small degree with microbiological analysis of jejunal aspirates (sensitivity 27%-52%, specificity 36%-80%)[70].

Scleroderma

Scleroderma (systemic sclerosis) is a chronic connective tissue disease that affects the gastrointestinal tract in more than 80% of patients[71]. Severe small bowel involvement by scleroderma can present as chronic intestinal pseudo-obstruction and SIBO. The reported prevalence of SIBO in scleroderma was 43% to 56%[72,73]. In our series, SIBO was proved in 4/15 (27%) patients with systemic sclerosis by means of glucose hydrogen and methane breath tests. Half the cases of SIBO had neither diarrhoea nor other signs of malassimilation at the time of examination. There was a tendency towards a higher dose of systemic glucocorticosteroids in persons with positive hydrogen and methane breath tests[74].

Autonomic neuropathy in diabetes mellitus

Gastrointestinal symptoms are present in 50%-70% of patients with diabetes mellitus. Delayed gastric emptying (or even diabetic gastroparesis) and intestinal motility disorders are the most important findings (with an unfavourable impact on glycaemic control). Impaired intestinal motility is often followed by SIBO[75-77]. In diabetes mellitus, first and foremost all results must be interpreted according to the diagnostic method that was used. Cuoco *et al*[78] performed the lactulose hydrogen breath test and found that 21/74 (28%) of subjects were affected by SIBO and delayed oro-caecal transit time. After treatment with rifaximin, three patients still showed SIBO, five persistent delayed transit time without SIBO and 13 persons (62%) experienced a significant improvement in their oro-caecal time (without SIBO)[78]. Reddymasu *et al*[79] used hydrogen and methane breath tests after glucose challenge. Thirty out of fifty (60%) patients had a positive breath test result for SIBO on the basis of hydrogen (63%), methane (27%) or both criteria (10%). SIBO was more likely in diabetic patients with gastroparetic symptoms of longer duration[79].

In about one third of patients with diabetes, SIBO was associated with cardiovascular autonomic neuropathy[77]. SIBO in diabetes may rarely manifest itself as protein-losing enteropathy[80].

Radiation enteropathy

SIBO and lactose intolerance may occur during and/or after pelvic (or abdominal) radiotherapy[81-83].

Fibromyalgia

Pimentel *et al*[14] found that 42/42 (100%) patients with fibromyalgia had an abnormal lactulose hydrogen breath test. This was a significantly higher rate compared to patients with irritable bowel syndrome (93/111, 84%) and clinically healthy persons used as a control (3/15, 20%). Patients with fibromyalgia also had a higher hydrogenic profile that correlated with somatic pain[14].

Other disorders and diseases associated with SIBO

Various diseases and disorders have been described to be associated with or complicated by SIBO, such as lymphoproliferative diseases (lymphoma, chronic lymphocytic leukaemia), benign lymphoid hyperplasia of the ileum, metabolic bone disease, acromegaly, hypothyreosis, alcoholism and rosacea[7,84-87]. The prevalence of SIBO rises with age (about 50% in persons > 75 years old)[88].

MICROBIOLOGY, PATHOGENESIS, PATHOPHYSIOLOGY AND PATHOLOGY

The total bacterial count in the proximal jejunum is < 10^4 bacteria per mL of jejunal content in healthy people. In the ileum, enteric bacterial populations increase in amount (including coliforms) up to 10^9 CFU/mL in the terminal ileum. There are several beneficial effects of normal small intestinal bacteria to the host. They can be extrapolated from experimental studies in germ-free animals. The small intestinal villi of these animals are thin and unusually regular, with relatively shortened crypts. The enterocytes are cuboidal rather than columnar. In addition, the number and size of Peyer's patches, the degree of leukocyte infiltration in the lamina propria, and the rate of mucosal regeneration are reduced. The introduction of micro-organisms rapidly restores the normal morphologic appearance and physiologic function of the small bowel mucosa[7].

Normal autochthonous bacterial flora of the gastrointestinal tract is an important factor for preservation of its integrity and normal functioning in humans. They participate in the protection of macro-organisms against pathogenic micro-organisms, stimulate the human immune system and influence the metabolic and trophic function of the intestinal mucosa. Enteric bacteria produce some nutrients (e.g. short-chain fatty acids) and vitamins such as folates and vitamin K. Last but not least, they impact the sensor and motor function of the gut. On the other hand, intestinal bacteria are influenced by many factors, first of all by the amount and composition of food, but also by environmental (and geographic) effects, drugs, alcohol and probably by several other factors (lifestyle, psychosomatic stress, *etc.*)[5,89]. The prevalence of bacteria in different parts of the GI tract appears to be dependent on several factors, such as pH, peristalsis, redox potential, bacterial adhesion, bacterial co-operation and antagonism, mucin secretion, diet and nutrient availability[90].

There are several host defence mechanisms to prevent excessive colonisation of the small bowel by bacteria: antegrade peristalsis prevents attachment of ingested micro-organisms; gastric acid and bile destroy many micro-organisms before they leave the stomach; digestion by proteolytic enzymes helps destroy bacteria in the small intestine; the intestinal mucus layer traps bacteria; an intact ileo-caecal valve inhibits retrograde translocation of bacteria from the colon to the small bowel; the immune system plays a role as evidenced by the high prevalence of bacterial overgrowth in patients who have immunodeficiency; the largest fraction of immunoglobulins secreted in the human body is the secretory IgA originating in the gastrointestinal tract, which aids in preventing bacterial proliferation[7,91]. SIBO may develop if some of the natural defensive mechanisms of a macro-organism (listed above) are disrupted.

In most patients, SIBO is not caused by a single bacterial strain. In general, there is an extension of colonic bacteria into the small bowel. Less frequently, the "normal" amount of small intestinal bacteria increases. Bouhnik *et al*[92] investigated samples of jejunal juice in 63 patients with diarrhoea and/or malabsorption. The diagnostic criteria of SIBO were fulfilled in 55 persons (87%). The authors identified 141 micro-aerophilic strains (*Streptococcus* 60%, *Escherichia coli* 36%, *Staphylococcus* 13%, *Klebsiella* 11% and others) and 117 anaerobes (*Bacteroides* 39%, *Lactobacillus* 25%, *Clostridium* 20% and others)[92].

SIBO may be accompanied by both maldigestion and malabsorption. Bacteria in SIBO might significantly interfere with enzymatic, absorptive and metabolic actions of a macro-organism. Due to injury of the brush-border of enterocytes, the activity of disaccharideses may be decreased. If bacteria simultaneously metabolise fructose, lactose and sorbitol, malabsorption of saccharides may occur. Injured small intestinal mucosa can have undesirable consequences in increased intestinal permeability and/or protein-losing enteropathy. Deficiency of vitamin B_{12} results from the consumption of this vitamin by anaerobic micro-organisms. Bacteria may also utilise intraluminal protein in the small bowel, this may lead to protein deficiency for the macro-organism and excessive production of ammonia by bacteria. Deconjugation of bile acids by bacteria results in malabsorption of fat and liposoluble vitamins. Extensively formed lithocholic acid is poorly absorbable and acts enterotoxically[5,7,93-95].

Bacteria produce various toxic agents that may have surprising systemic effects. These agents are ammonia, D-lactate, endogenous bacterial peptidoglycans and others. SIBO is regularly associated with increased serum endotoxin and bacterial compounds stimulating production of (pro)inflammatory cytokines[7,96]. SIBO might be associated with endogenous production of ethanol (probably synthesised by *Candida albicans* and *Saccharomyces cerevisiae*). Serum ethanol disappears after successful treatment of SIBO[37].

Small intestinal bacterial overgrowth has a negative impact not only on the function but also on the morphological structure of the small bowel. Microscopic inflammatory changes (especially in the lamina propria) and villous atrophy are found regularly. In such a case, the villous atrophy in SIBO must be distinguished from that of coeliac disease. Macroscopic changes may also be visible in some patients. Hoog *et al*[97] found small intestinal mucosal breaks (erosions or ulcers) in 16/18 patients with chronic myopathic or neuropathic motility disorders of the small bowel by means of wireless capsule endoscopy.

In some patients with short bowel syndrome, bacterial overgrowth can, to some extent, paradoxically exert a favourable effect on the macro-organism. Bacteria may partly metabolise saccharides and thus form some further energy substrates more easily utilisable by a diseased human.

CLINICAL FEATURES

Clinical symptoms are expressed more or less according to the severity of involvement and they are modified by a primary underlying disease. SIBO may be clinically asymptomatic or can resemble irritable bowel syndrome with

non-specific symptoms (bloating, flatulence, abdominal discomfort, diarrhoea, abdominal pain). In more severe cases, there are signs of malabsorption (weight loss, steatorrhoea, malnutrition), liver lesion, skin manifestation (rosacea), arthralgias and deficiency syndromes (anaemia, tetany in hypocalcaemia induced by vitamin D deficiency, metabolic bone disease, polyneuropathy due to vitamin B_{12} deficiency, impaired barrier function of the gut, *etc.*). Anaemia is usually macrocytic (megaloblastic) due to vitamin B_{12} deficiency. It could also be microcytic iron deficiency (due to occult gastrointestinal blood loss) or normocytic (as anaemia of chronic disease)[3,5-7]. Serum folate and vitamin K levels are usually normal. Serum vitamin K can even be increased owing to its bacterial overproduction. Moreover, there are some concerns as to whether endogenous intestinal production of vitamin K by bacteria might interfere with warfarin treatment in SIBO[98-100]. In the case of oedema of lower extremities, the aetiology is usually more complex (anaemia, malnutrition, hypoproteinaemia, vitamin B_{12} deficiency).

D-lactic acidosis is a severe complication of patients with short bowel syndrome (with intact large bowel). It is caused by an excessive overgrowth of lactobacilli. Non-absorbed saccharides pass from the small intestine to the large bowel and they are fermented down to the D-isomer of lactic acid. There is no human pathway to metabolise D-lactic acid. D-lactic acid is absorbed from the large bowel; its serum concentration is regularly increased in these patients. Nevertheless, most patients remain asymptomatic. In clinically expressed cases, leading symptoms comprise characteristic neurologic abnormalities including confusion, cerebellar ataxia, slurred speech, and loss of memory. Patients exhibit some degree of altered mental status. They may complain of or appear to be drunk in the absence of ethanol intake. In the treatment, it is necessary to compensate metabolic acidosis and administer peroral antibiotics (metronidazole, rifaximin). To prevent this serious complication, it is important to reduce peroral intake of simple sugars, polysaccharides given in smaller amounts together with a higher intake of fat[101].

DIAGNOSTICS

It is mandatory to consider SIBO in all cases of complex non-specific dyspeptic complaints (bloating, abdominal discomfort, diarrhoea, abdominal pain), in motility disorders, anatomical abnormalities of the small bowel and in all malassimilation syndromes (malabsorption, maldigestion)[3,5-7].

Physical investigation usually provides non-specific findings and could be modified by a primary underlying disease. The abdomen may be distended and a small intestinal succussion splash might be identified. Physical investigation can further reveal latent tetany, polyneuropathy and skin manifestation (rosacea).

Laboratory tests usually find anaemia, low serum vitamin B_{12} levels and laboratory signs of malnutrition (lymphopenia, low serum prealbumin and transferrin).

The gold standard for diagnosing SIBO is still microbial investigation of jejunal aspirates. Such a sample can be obtained by a special sonde or by means of enteroscopy. Nowadays, there are commercially available special aspiration catheters (with a spiral pattern of holes at the distal tip) for contaminate-free collection of fluids. Microbial investigation places high demands on the quality of laboratory work (determination of quantitative proportion of anaerobes) and has several difficulties (low reproducibility and identifying cultivation-resistant bacteria). Distribution of bacterial overgrowth might be irregular and that is why a single investigation might not detect it. Bacterial overgrowth may be restricted to a particular, difficult-to-access area for aspiration (e.g. a blind loop)[7].

Hydrogen and methane breath tests are currently the most important diagnostic methods. The principles and methods of hydrogen and methane breath tests were described in detail elsewhere[102-105]. In humans, hydrogen and methane are exclusively produced by intestinal bacteria, namely in the large bowel in healthy people and also in the small intestine in the case of SIBO. About 80% of hydrogen and methane is expelled by flatus, 20% is exhaled by lungs and can be measured in breath[106]. Hydrogen and methane breath tests to diagnose SIBO are performed after peroral glucose or lactulose challenge. Most authors (including our Department) use gas chromatography for breath analysis. A parallel measurement of CO_2 and correction of hydrogen values to CO_2 concentration make the measurement more precise[7,74,103,104]. Low humidity (< 25%) must be maintained in the laboratory atmosphere to obtain consistent measurements. There is an early increase in breath hydrogen and/or methane (single early peak) after glucose administration due to bacterial glucose fermentation in the small bowel. There are two peaks in the lactulose breath test, the first one owing to bacterial activity in the small intestine, the second one after lactulose reaches the colon. Unfortunately, hydrogen and methane breath tests have not yet been standardised, particular protocols differ in dose (and concentration) of the test substrate, duration of tests, time intervals of breath sampling and basic and peak cut-off values. According to most authors, basal cut-off values of hydrogen and/or methane in positive breath tests are $\geqslant$ 20 parts per million (ppm), 10-20 ppm is a grey zone. After a glucose challenge, an increase $\geqslant$ 12 ppm at 120 min is a positive result for bacterial overgrowth. A lactulose breath test is assessed as positive if there is a biphasic course or an early plateau pattern with a hydrogen increase of $\geqslant$ 12 ppm is found (possibly with an increase in methane at the second peak)[104,107-110].

The hydrogen breath test is considered to be more accurate for the diagnosis of SIBO compared to the methane breath test according to most authors[111-114]. There is a sensitivity of 62.5% and specificity of 82% (diagnostic accuracy of 72%) after glucose and 52% and 86% (diagnostic accuracy of 55%) after lactulose administration[109]. Hydrogen alone, methane alone or both gases simultaneously might be found in breath samples. That is why it is important to always determine both gases in the breath samples. There are several advantages of hydrogen and methane breath tests. They are non-invasive, non-toxic,

relatively easily available and performed at a low cost.

However, hydrogen and methane breath tests have some drawbacks with possible false results and difficulties in their interpretation. Very rapid absorption of glucose in the proximal jejunum can be responsible for a false negative result. In the case of bacterial overgrowth in the terminal ileum, it might be difficult to distinguish a pathological ileal peak from a "normal peak" after the caecum is reached. In short bowel syndrome (with intact large bowel), the test substrate may reach the colon very quickly and cause a false positive result. In the case of a low density of anaerobes, breath can be false negative[109,113,115-117]. If there is only one peak of hydrogen recorded in the lactulose breath test and the second peak is absent for hydrogen and methane, this could be assessed as a combination of SIBO and fermentative colopathy[108]. Results of hydrogen and methane breath tests are usually difficult to interpret in the case of advanced lung disease. In the case of a high concentration of hydrogen and low concentration of methane, analytical precision for methane determination is less accurate[104].

All hydrogen and methane are produced by so called "hydrogenic and methanogenic" bacteria in humans[103,104,106,118]. However, most authors usually do not specify which particular bacteria constitute these producers. Nitrogen, oxygen, carbon dioxide, hydrogen and methane account for more that 99% of expelled intestinal gas. Hydrogen is produced by bacterial fermentation of saccharides in the intestinal lumen. Concurrently, hydrogen is consumed by other intestinal bacteria to synthesise methane, acetate and hydrogen sulphide. Methane is synthesised solely by bacteria in the intestine (four mmols of hydrogen and one mmol of carbon dioxide create one mmol of methane and water). This reaction reduces the volume of gas that would otherwise be present in the colon[119-124]. The question of intestinal methane producers has not been definitely solved yet. *Methanobrevibacter smithii*, *Methanosphaera stadtmanae* and other *Methanobacteriales* are able to synthesise methane and some authors consider *Methanobrevibacter* to be the major producer of methane in the gut of humans[125,126]. It was assumed (based on 16S ribosomal DNA studies) that *Methanobrevibacter smithii* could make up about one in ten of all the prokaryotes in the human gut[127]. However, there is no final proof available that *Archea* (*Methanobrevibacter* and others) would be the prevailing microorganisms in the methanogenic phenotype of the human gut. We hypothesised that common coliform bacteria could also synthesise methane[128], however, this assumption was not proved by our further studies[129,130]. McKay *et al*[131] found that several anaerobes (*Bacteroides*, *Clostridium* and others) produced hydrogen but rarely methane. Hydrogen is also produced by *Enterobacteriaceae*[128,132].

In adult Caucasians, only 30%-50% of persons produce methane while hydrogen is produced by 90%-98% of people[104]. Most subjects with lactose intolerance who do not produce hydrogen would form methane after lactulose administration instead of hydrogen[133]. Bile in the intestinal lumen is an important suppressor of methanogenesis in humans[134]. According to some authors, a methanogenic phenotype is associated with constipation[8,12]. Methane production has been related to more severe colonic impaction in children with encopresis[135]. Higher production of methane has been detected in colorectal adenomas and cancer[136]. However, there is a general agreement that constipation itself is not a risk factor for the development of colorectal cancer.

Hydrogen and methane breath tests can be combined with a simultaneous D-xylose breath test[107,110]. This combination increases the sensitivity of non-invasive diagnostics of SIBO[107,114]. The cholyl-1-^{13}C-glycine hydrolase breath test is another possible alternative for the diagnosis of SIBO. The principle of this test is based on the fact that bacterial overgrowth will cause more rapid deconjugation of cholyl-1-^{13}C-glycine[137]. The reported sensitivity for this test was 70%[138]. The lack of sensitivity was attributed to bacterial overgrowth with species lacking cholylglycine hydrolase[137]. On the other hand, patients with bile acids malabsorption in the ileum might have a false positive result in this breath test after rapid deconjugation of cholylglycine in the proximal colon[139]. Berthold *et al*[140] recently proposed a lactose-^{13}C-ureide breath test for the diagnosis of small bowel bacterial overgrowth. This test should have 100% specificity (and thus predict positive culture in SIBO) but lower sensitivity (66%)[140].

There are several other tests to diagnose SIBO, for instance evaluation of short-chain fatty acids in jejunal aspiration[141], serum non-conjugated bile acids, urinary output of p-aminobenzoic acid (after peroral administration of colil-PABA) or urinary indican[6]. However, none of these tests has yet acquitted itself well in routine clinical practice.

If it is impossible to perform any diagnostic test for SIBO (if no test is available for a particular department) on a patient with legitimate suspicion of SIBO, it is possible to consider the exceptional use of an empiric therapeutic test with rifaximin (for 7-10 d). Quick disappearance of symptoms supports a possible diagnosis of SIBO, however, this is not definite outright proof of SIBO. On the other hand, demonstration of SIBO is not 100% proof of causal association between bacterial overgrowth and clinical symptoms (or laboratory abnormal results).

In some patients with SIBO, secondary inflammatory changes might be found not only in the small bowel but also in the colon as a response to absorbed bacterial antigens. This inflammatory involvement can cause separate symptoms[7]. Successful treatment with 5-aminosalicylates and glucocorticosteroids supports this theory[142].

DIFFERENTIAL DIAGNOSIS

Diagnosis and differential diagnosis of SIBO is difficult if this possibility is not considered. It is necessary to distinguish functional disorders (of no organic cause) and chronic gastrointestinal infections (e.g. giardiasis).

The relationship between SIBO and irritable bowel syndrome was discussed above. Esposito *et al*[44] proposed use of the lactulose breath test to distinguish SIBO and

irritable bowel syndrome. Parodi *et al*[143] recommend differentiating patients fulfilling the diagnostic criteria of irritable bowel syndrome (IBS-like symptoms) from functional bloating. If SIBO is proved, the first group will profit from antibiotic treatment while the second group will not[143].

It is always necessary to consider SIBO in the case of unexplained deterioration of the clinical status of patients with Crohn's disease, chronic pancreatitis or scleroderma. SIBO must be taken into account in coeliac disease non-responding to adequate gluten-free diet. SIBO is a crucial point in the differential diagnosis of short bowel syndrome and all other malassimilation syndromes (both with maldigestion and malabsorption).

On the other hand, some other intestinal disorders might mimic SIBO and must be considered in the differential diagnosis. Flatulence, abdominal bloating and distension, and malabsorption of mono- or disaccharides (like fructose or lactose) must be taken into account. Pneumatosis cystoides intestinalis is usually asymptomatic but it may be associated with abdominal pain, bloating and/or diarrhoea[106].

PRINCIPLES OF TREATMENT

Therapy for SIBO must be complex (addressing all causes, symptoms and complications) and fully individualised. It should include treatment of the underlying disease, nutritional support and cyclical gastro-intestinal selective antibiotics.

The most important thing is always treatment of the basic underlying disease if possible. Nutritional support is mandatory in SIBO associated with malnutrition, weight loss and nutrient deficiency. We usually use individualised diet, enteral nutrition by fine-bore naso-jejunal tube or nutritional support by sipping of polymeric formulas. In several patients, it is necessary to exclude lactose from the diet, to reduce other simple sugars, to increase coverage of energy needs by fat and to administer MCT oils (medium-chain triacylglyceroles).

Antibiotic treatment should selectively target those bacterial strains that cause SIBO. The choice of antibiotics should be based on sensitivity testing to particular antibiotics. However, this requirement is difficult to achieve in clinical practice as various bacteria are usually found simultaneously, each with different sensitivity to antibiotics. There is no common agreement concerning choice, dosing and duration of antibiotic therapy. In general, long-term treatment with broad-spectrum antibiotics is not the optimal solution as such a therapy is associated with several problems (intolerance by the patient, dysmicrobia, diarrhoea, *Clostridium difficile* expansion, possible increased resistance to antibiotics, financial cost, *etc.*).

Tetracycline was considered the treatment of choice for a long time. Di Stefano *et al*[144] administered tetracycline to patients with SIBO for 7 d (1000 mg/d) and achieved normalisation of the hydrogen breath test together with relief of symptoms in only 3/11 (27%) subjects[144]. Various antibiotics were tried in other small clinical studies. Attar *et al*[145] administered a placebo, norfloxacin (800 mg/d), amoxicillin clavulanate (1500 mg/d) and *Saccharomyces boulardii* (1500 mg/d) successively in 7-d intervals in 10 patients. Norfloxacin and amoxicillin clavulanate significantly decreased the frequency of diarrhoea compared to the placebo (in 9/10 and 6/10 patients, respectively), but the hydrogen breath test was normalised in only 3 and 5 subjects[145]. In Crohn's disease, Castiglione *et al*[56] achieved normalisation of the hydrogen breath test in 13/15 (87%) persons treated with metronidazole (750 mg/d) and in 14/14 (100%) treated with ciprofloxacin (1000 mg/d)[56]. Di Stefano *et al*[146] divided 21 patients with a blind loop syndrome into three different treatment groups: (1) rifaximin followed by metronidazole, or (2) two courses of metronidazole, or (3) two courses of rifaximin. Both antibiotics were effective; metronidazole markedly reduced both hydrogen breath tests and patients' symptoms[146]. However, rifaximin was more effective than metronidazole in another study (63% *vs* 44%)[147]. A drawback in all of these studies was not only the small set of patients but also the absence of long-term follow-up. Pimentel *et al*[12] administered neomycin or a placebo to 111 patients with irritable bowel syndrome (84% abnormal lactulose breath test). Neomycin improved both symptoms and the breath test in 35% of persons compared with 11% in the placebo group[12]. Some other peroral antibiotics, such as cephalexin, trimethoprim-sulfamethoxazole, levofloxacin and gentamicin were used for the therapy of SIBO[7].

The greatest experience for treatment of SIBO was acquired with rifaximin[43,148-154]. Rifaximin is a semi-synthetic rifamycin-based non-systemic antibiotic, with a low gastrointestinal absorption and good bactericidal activity. The antibacterial action covers Gram-positive and Gram-negative organisms, both aerobes and anaerobes[155]. According to different studies, rifaximin improves symptoms in 33%-92% and eradicates small intestinal bacterial overgrowth in up to 80% of patients[151,152]. Most authors recommend administering rifaximin for 7-10 d as one treatment course or as a cyclic therapy. Higher doses (1200 or 1600 mg/d) are more effective than standard doses (600 or 800 mg/d)[148,154]. Rifaximin is probably the only antibiotic that is capable of achieving a long-term favourable clinical effect in patients with irritable bowel and SIBO[43].

Prebiotics and probiotics exert various beneficial effects in the macro-organism, they strengthen the barrier function of the gut, inhibit several pathogens, modify the inflammatory response of the bowel, and they also reduce visceral hypersensitivity[156-159]. They seem to be more effective in influencing the clinical symptoms of irritable bowel syndrome compared to a placebo[159,160]. Studies dealing with the therapeutic use of prebiotics or probiotics in SIBO (except irritable bowel syndrome) are limited[161-163], and it is not therefore possible to recommend them for general clinical use[157,158]. *Lactobacilli*-based probiotics are contraindicated in patients with a risk of D-lactic acidosis. Very little data are available from experimental studies. Short-term administration of the hydrogenic probiotic *Escherichia coli 1917 Nissle* (3.5×10^{10} bacteria per

day for 14 d) did not influence methanogenic phenotype of experimental non steroidal anti-inflammatory drug-enteropathy in pigs[129].

Prokinetics seem to be a logical therapeutic step in SIBO due to motility disorders. Several studies tried metoclopramide, cisapride (which was later withdrawn from the market), domperidone, erythromycin, itopride, tegaserod and octreotide. However, there are only limited data suggesting that this treatment would be effective over the long term[7,71]. Cyclic lavages of the small bowel (e.g. by polyethylene glycol) can be considered as supportive therapy in cases of relapsing SIBO[7].

Surgical treatment must always be considered where possible to correct gastrointestinal pathology (entero-colic fistulae, blind loops, bowel obstruction, multiple small intestinal diverticula, *etc.*). Specialised non-transplant surgery can provide interventions in short bowel syndrome improving intestinal motility (STEP - serial transverse enteroplasty), slowing intestinal transit (valves, reversed segments, colon interposition) or increasing mucosal surface area of the gut (creation of "neo-mucosa", sequential intestinal lengthening)[164].

PROGNOSIS

The prognosis of SIBO is determined mostly by the underlying disease leading to bacterial overgrowth. Ultimately SIBO might result in intestinal failure[61]. In scleroderma with gastrointestinal involvement (SIBO, intestinal pseudo-obstruction, malnutrition), the overall 5-year mortality is more than 50%[71].

The relapse rate of SIBO after successful treatment is high. Lauritano *et al*[165] found recurrence of SIBO in 44% (35/80) of patients nine months after successful treatment with rifaximin. Apart from the basic underlying disease, further risk factors for recurrence of SIBO have been identified including older age (OR 1.1), appendectomy in the patient's history (OR 5.9) and long-term treatment with proton pump inhibitors (OR 3.5)[165].

CONCLUSION

SIBO is defined as an increase in the number and/or alteration in the type of bacteria in the upper gastrointestinal tract. The aetiology of SIBO is usually complex, associated with disorders of protective antibacterial mechanisms (e.g. achlorhydria, pancreatic exocrine insufficiency, immunodeficiency syndromes), anatomical abnormalities (e.g. small intestinal obstruction, diverticula, fistulae, surgical blind loop, previous ileo-caecal resections) and/or motility disorders.

SIBO is often misdiagnosed and generally underdiagnosed. Clinical symptoms might be non-specific (dyspepsia, bloating, abdominal discomfort). Nevertheless, SIBO can cause severe malabsorption, serious malnutrition and deficiency syndromes. Non-invasive hydrogen and methane breath tests after glucose or lactulose challenge are most commonly used for the diagnosis of SIBO. Therapy of SIBO must be complex and should include treatment of the underlying disease, nutritional support and cyclical gastrointestinal selective antibiotics. Prognosis is usually serious, determined mostly by the underlying disease that led to SIBO.

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OBSERVATION

Mesenteric lymph node cavitation syndrome

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Abstract

The mesenteric lymph node cavitation syndrome consists of central necrosis of mesenteric lymph nodes and may occur with either celiac disease or a sprue-like intestinal disease that fails to respond to a gluten-free diet. Splenic hypofunction may also be present. The cause is not known but its development during the clinical course of celiac disease is usually indicative of a poor prognosis for the intestinal disorder, a potential for significant complications including sepsis and malignancy, particularly T-cell lymphoma, and significant mortality. Modern abdominal imaging modalities may permit earlier detection in celiac disease so that earlier diagnosis and improved understanding of its pathogenesis may result.

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Key words: Mesenteric lymph node; Celiac disease; Yersinia infection; Tuberculosis; Kikuchi disease; Hyposplenism; Lymphoma

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INTRODUCTION

Over 50 years ago, mesenteric lymph node changes were described in celiac disease[1-3]. At that time, definition of most changes in abdominal lymphoreticular structures relied largely on surgical observation or postmortem evaluation. In addition, altered abdominal lymphoreticular function was also evident, reflected in reduced splenic function[4-6]. In recent years, refinements in abdominal imaging, i.e. ultrasound, computed tomography (CT), magnetic resonance imaging (MRI) and positron emission tomography, have led to improved and earlier documentation of lymphoreticular changes in celiac disease. Neoplastic (i.e. lymphoma) and non-neoplastic changes, including mesenteric lymph node cavitation, have been recorded.

MESENTERIC LYMPH NODE CAVITATION SYNDROME

Detailed descriptions of this entity appeared largely in the French literature[7-9]. These reports described an unusual syndrome that seemed to complicate the clinical course of adult celiac disease. While typical small intestinal mucosal biopsy changes of celiac disease were present, the most intriguing observation was the definition of cavitated mesenteric lymph nodes. In addition, splenic atrophy or splenic hypofunction appeared to be present, raising the spectra of impaired immune function and a specific infectious cause.

Matuchansky and collegues[10], in a seminal report, noted details of 6 cases that included 4 females and 2 males. Within the jejunoileal mesenteric nodes, pseudocystic lesions were detected that consisted histologically of a large central cavity occupied by hyaline-type material surrounded by fibrous tissue and lymph node remnants. Evidence of mesenteric panniculitis or malignant lymphoma was not detected. Persistent diarrhea or childhood diarrhea along with malabsorption accompanied the small intestinal changes. An unequivocal mucosal response to a gluten-free diet was shown in some cases confirming that celiac disease was present. In others, a definitive response to a gluten-free diet could not be documented suggesting

that either a resistant form of celiac disease or sprue-like intestinal disease was present. Similar features have been noted with other complicating disorders (e.g. lymphoma) in both adult celiac disease or sprue-like intestinal disease[11]. Microscopic examination of the lymph nodes in these cases usually shows some common features. Atrophic cavitated lymph nodes are usually present without evidence of infection or malignancy in the lymph nodes. Central acidophilic lipid-containing fluid is usually present surrounded by a rim of residual lymph node tissue that includes follicles, sinus and capsular components. The changes historically have appeared to be confined to the mesenteric lymph node chain.

HYPOSPLENISM

Another important, although not essential[4-6], component of this clinical syndrome appears to be splenic atrophy or splenic hypofunction. While splenic changes have been previously noted in other intestinal disorders (e.g. inflammatory bowel disease)[12], mesenteric lymph node cavitation was seen only in those with pathological changes of celiac disease or sprue-like small intestinal disease, and not with inflammatory bowel disease[10]. In early studies, splenic atrophy was often defined structurally during postmortem evaluation or abdominal surgery. More recently, these changes tended to be defined with modern imaging methods. Hyposplenism, indicative of reduced splenic function, appeared to reflect this structural change in the spleen, and depending on its mode of measurement, seemed to be quite common in celiac disease[6]. The latter disorder may be suspected from a peripheral blood smear with Howell-Jolly bodies, monocytosis, lymphocytosis and increased platelet counts, and confirmed with pitted red blood cells counts or radiolabeled colloid scanning of the spleen[13-15]. In celiac disease, for example, 76.2% of patients were found to have pitted red blood cells[6]. These may also occur during the clinical course of many other disorders, apart from celiac disease or sprue-like intestinal disease[13-15]. These disorders include dermatitis herpiformis[16], collagenous sprue[17], collagenous colitis[18] and abdominal lymphoma[14,19], all disorders intimately linked to celiac disease[19-21]. Hyposplenic patients have been reported to be at increased risk for bacterial sepsis, especially with encapsulated organisms such as pneumococcus, sometimes with a fatal outcome[22], so vaccination using the pneumococcal conjugate has been recommended[14]. The risk of sepsis appears to be more significant if a diagnosis of celiac disease is established during adult years rather than in childhood[23]. There may also be a higher risk for vascular, autoimmune and thrombotic disorders along with solid tumors[14]. In celiac disease with associated autoimmune or malignant complications, IgM memory B-cells were reduced suggesting a possible mechanism leading to infections by encapsulated bacteria[14]. Resolution of reduced splenic function appears to be possible, but only after improvement of small intestinal mucosal changes has occurred[6].

LONG-TERM NATURAL HISTORY AND COMPLICATIONS

Most early reports indicated that cavitation of mesenteric lymph nodes was associated with a very poor prognosis with a mortality of about 50%[24]. Nevertheless, dramatic improvement has also been recorded with complete normalization of the mesenteric lymph nodes[25,26].

In celiac disease with associated dermatitis herpetiformis, recurrent diarrhea, steatorrhea and protein-losing enteropathy were defined followed later by hyposplenism, mesenteric lymph node cavitation and malignant lymphoma of the small intestine[27]. In another case of mesenteric lymph node cavitation, lymphoma also developed with a sprue-like intestinal lesion that did not respond to a gluten-free diet[28]. Cavitation of mesenteric lymph nodes may also occur without celiac or other small bowel disease[29]. Bacterial infections may be associated with necrosis of lymph nodes (e.g. mycobacteria, *Yersinia*) and lymph node cavitation has been reported with Whipple's disease[30]. Necrotizing lymphadenitis may also occur with systemic lupus erythematosus[31], and this appears to be pathologically similar to Kikuchi-Fujimoto disease, a self-limited form reported in young Asian adults[32]. In celiac disease, however, malignant lymphoma may also be the cause of necrotic mesenteric lymph node changes[33]. In one report, these necrotic lesions were seen in the liver and spleen due to hepatosplenic type lymphoma, a rare type of peripheral T-cell lymphoma with T-cell receptor rearrangement[33]. In a more recent case report, progressive encephalopathy and focal lesions of the cerebellum and brainstem were associated with abnormal T-cell clones in a sprue-like disorder with mesenteric and mediastinal lymph node cavitation[34]. Finally, a case of necrotizing hepatitis with celiac disease and mesenteric lymph node cavitation was recently described, but lymphoma was not detected[35].

CONCLUSION

This syndrome is an intriguing entity or group of entities frequently associated with celiac disease or sprue-like intestinal disease consisting of cavitated mesenteric lymph nodes and, often, splenic hypofunction. Cavitated mesenteric nodes should be differentiated from other causes of mesenteric cystic lesions, including lymphatic cysts or cystic lymphangioma. These have also been recorded with celiac disease and hyposplenism[36]. Although diagnosis is usually confirmed by pathological evaluation of mesenteric lymph nodes, imaging studies may be very helpful. Ultrasound may show anechoic cysts up to 8 cm in size. CT may reveal low attenuation (fluid or fat) lymphadenopathy suggesting celiac disease, Whipple's disease, infections such as tuberculosis, lymphoma or necrotic metastases, including germ cell tumors[37]. Occasionally, fluid-fat levels are appreciated in the lymph node, reported only in celiac disease[37]. MRI may be useful as the fluid and fat layer may be appreciated in T2- and T1-weighted axial images, even if fluid-fat levels are not seen on CT[38]. In some cases, image-guided fine-needle aspiration of the

cyst may be performed[38]. The cause of cavitation syndrome is usually not defined. The mesenteric lymph node cavitation changes have been hypothesized to represent excess antigen exposure via damaged small bowel mucosa causing lymphoid cell depletion in the lymph nodes and spleen. Alternatively, changes may reflect necrosis in the mesenteric nodes triggered by localized immune-mediated complement activation and intravascular coagulation[38]. Specific infections and malignant lymphoma may complicate its natural history and clinical course and contribute to its poor prognosis.

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ORIGINAL ARTICLE

Emodin enhances alveolar epithelial barrier function in rats with experimental acute pancreatitis

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Abstract

AIM: To investigate the effect of emodin on expression of claudin-4, claudin-5 and occludin, as well as the alveolar epithelial barrier in rats with pancreatitis induced by sodium taurocholate.

METHODS: Experimental pancreatitis was induced by retrograde injection of 5% sodium taurocholate into the biliopancreatic duct. Emodin was injected *via* the external jugular vein 3 h after induction of acute pancreatitis. Rats from sham operation group and acute pancreatitis group were injected with normal saline (an equivalent volume as emodin) at the same time point. Samples of lung and serum were obtained 6 h after drug administration. Pulmonary morphology was examined with HE staining. Pulmonary edema was estimated by measuring water content in lung tissue samples. Tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) level were measured by enzyme-linked immunospecific assay. Serum amylase and pulmonary myeloperoxidase (MPO) activity were detected by spectrophotometry. Alveolar epithelial barrier was assessed by pulmonary dye extravasation. Expression of claudin-4, claudin-5 and occludin in lung tissue samples was examined by immunohistology, quantitative real-time reverse transcription polymerase chain reaction and Western blotting analysis, respectively.

RESULTS: Pancreatitis-associated lung injury was characterized by pulmonary edema, leukocyte infiltration, alveolar collapse, and elevated serum amylase level. The pulmonary damage, pulmonary pathological scores, serum amylase and MPO activity, TNF-α and IL-6 levels, and wet/dry ratio were decreased in rats after treatment with emodin. Immunostaining of claudin-4, claudin-5 and occludin was detected in lung tissue samples from rats in sham operation group, which was distributed in alveolar epithelium, vascular endothelium, and bronchial epithelium, respectively. The mRNA and protein expression levels of claudin-4, claudin-5 and occludin in lung tissue samples were markedly decreased, the expression level of claudin-4, claudin-5 and occluding was increased, and the pulmonary dye extravasation was reduced in lung tissue samples from rats with acute pancreatitis after treatment with emodin.

CONCLUSION: Emodin attenuates pulmonary edema and inflammation, enhances alveolar epithelial barrier function, and promotes expression of claudin-4, claudin-5 and occludin in lung tissue samples from rats with acute pancreatitis.

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Key words: Acute pancreatitis; Emodin; Lung injury; Claudin; Occludin; Epithelial barrier

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INTRODUCTION

Acute pancreatitis is a common disease with a considerable morbidity and mortality of 20%[1,2]. Its mortality is attributed to inflammation-related complications, such as pancreatitis-associated lung injury, clinically presenting as adult respiratory distress syndrome[2-4]. Intervention can reduce its morbidity and mortality, although its mechanism remains unclear[4].

Pancreatitis-associated lung injury is characterized by significant pulmonary edema, hyperemia and inflammatory infiltration in alveoli[5]. It has been established that pulmonary edema is related to increased permeability and loss of barrier function[2-5]. Although elevated levels of pancreatic enzymes and pro-inflammatory cytokines are attributed to pulmonary vasculature damage and increased endothelial permeability[6], the molecular basis for these damages remains largely undefined. Tight junctions are intimately involved in epithelial and endothelial permeability[7]. Fernandez *et al*[8] recently demonstrated that claudins, the key components of tight junctions, restrict the paracellular movement of water, proteins, and solutes across cellular barriers including alveolar epithelium. In mammals, the claudin family includes at least 24 members. With small interfering RNA and a blocking peptide, Wray *et al*[9] described that inhibition of claudin-4 decreases transepithelial electrical resistance in primary rat and human epithelial cells, as well as air space fluid clearance, resulting in pulmonary edema in mice, suggesting that claudin-4 plays an important role in alveolar epithelial barrier function. Moreover, claudin-5 and occludin are also decreased in models of acute lung injury accompanying increased paracellular permeability, indicating that claudin-5 and occludin may also play a role in alveolar epithelial barrier function[10-12]. However, the relation between expression of claudin-4, claudin-5, and occludin in lung tissues of patients with acute pancreatitis and pancreatitis-associated lung injury remains largely undefined.

It was reported that emodin (1,3,8-trihydroxy-6-methyl-anthraquinone), an anthraquinone derivative from the Chinese herb *Radix et Rhizoma Rhei*, inhibits the production of inflammatory cytokines such as tumor necrosis factor-α (TNF-α)[13]. Our previous study demonstrated that emodin significantly reduces serum TNF-α and interleukin-6 (IL-6) levels, thus attenuating lung injury in rats with acute pancreatitis[14]. The effect of emodin on pulmonary tight junction expression and alveolar epithelial barrier function, however, needs to be further defined.

In the present study, the effect of emodin on pancreatitis-associated lung injury and alveolar epithelial barrier function was assessed by examining pulmonary morphology, myeloperoxidase (MPO) activity (indicator of inflammatory infiltration), expression of claudin-4, claudin-5 and occludin, as well as dye extravasation, in lung tissue samples from rats with acute pancreatitis.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawely rats, weighing 200-250 g, obtained from Animal Facility of Jinling Hospital (Nanjing, China), were housed under controlled temperature and humidity in a day-night cycle, with free access to standard laboratory foot and water. The study was approved by Animal Studies Ethics Committee of Jinling Hospital.

Experiment model

Acute pancreatitis was induced as previously described[15]. Briefly, animals were anesthetized with intraperitoneal ketamine (80 mg/kg) and acepromazine (2.5 mg/kg). The biliopancreatic duct was cannulated through the duodenum, and the hepatic duct was closed with a small bulldog clamp. Pancreatitis was induced by retrograde injection of 5% sodium taurocholate (Sigma, St. Louis, MO, USA) into the biliopancreatic duct (1 mL/kg body weight), at a constant infusion pressure of 20 mmHg. Rats in sham operation group received retrograde sterile saline infusion.

Experiment design

Effect of emodin on expression of claudin-4, claudin-5 and occludin, as well as on pulmonary dye extravasation, a marker to evaluate alveolar epithelial barrier, was detected in rats with acute pancreatitis. Time course of pulmonary edema and inflammation was recorded. Rats with acute pancreatitis were randomly allocated into pancreatitis group and emodin treatment group. Rats in pancreatitis group were injected with emodin (2.5 mg/kg body weight) *via* the external jugular vein 3 h after sodium taurocholate infusion. Rats in sham operation group were injected with normal saline (equivalent volume as emodin) at the same time point and served as a control group.

Lung tissue samples were obtained 6 h after emodin injection, and maintained at -80℃ until assay. Blood samples were obtained from the inferior cava vein by direct puncture. Lung tissue samples were fixed in 4% neutral phosphate-buffered formalin and embedded in paraffin wax for histology examination. Serum amylase activity was detected to confirm the appropriate induction of pancreatitis.

Measurement of serum amylase level

Serum amylase level was measured by incubating serum with 4,6-ethylidene (G7)-p-nitrophenyl (G1)-1-D-maltoheptoside for 2 min at 37℃, with its absorbance detected once a minute for 2 min at 405 nm by high through universal microplate assay (BMG Lab Technologies, Germany).

Histological examination

Lung tissue sections were stained with hematoxylin and eosin. An experienced pathologist and a pancreatic specialist assessed tissue alterations under light microscope in a blinded fashion and scored them with a grading system[16]. The grading involved measurements of inflammatory infiltration, pulmonary edema and alveolar collapse, each on a scale of 0-3, giving a maximum score of 9.

Measurement of pulmonary cytokine level and MPO activity

TNF-α and IL-6 levels in lung tissue samples were measured using a sandwich enzyme-linked immunospecific assay (Jingmei Biotech, Beijing, China) according to its manufacturer's instructions. Absorbance was measured at 450 nm by high through universal microplate assay. Tissue homogenate was corrected with the protein concentration and expressed as per protein in lung tissue (pg/mg protein).

Sequestration of neutrophils in lung tissue samples was evaluated by measuring tissue MPO activity[15,17]. Briefly, lung tissue samples were homogenized with 0.5% hexadecyltrimethylammonium bromide (Sigma, St. Louis, MO, USA) in 50 mmol/L phosphate buffer (pH 6.0). Homogenate was sonicated for 10 s, freeze-thawed three times, and centrifuged at 14000 *g* for 15 min. The resulted suspension was used for assay. The assay mixture contained 20 μL of supernatant, 10 μL of tetramethylbenzidine (final concentration 1.6 mmol/L), and 70 μL of H_2O_2 (final concentration 3.0 mmol/L). MPO activity was assessed photometrically at 630 nm. The results were corrected with the protein concentration and expressed as the activity of per protein in lung tissue (U/mg protein).

Evaluation of pulmonary edema and alveolar epithelial barrier function

Severity of pulmonary edema was estimated by measuring water content in lung tissue samples. Freshly blotted lung tissue samples were weighed on an aluminum foil, dried for 24 h at 95℃, and reweighed. Difference in wet and dry tissue weights was calculated and expressed as wet/dry ratio.

Alveolar epithelial barrier function was evaluated by measuring Evans blue (Sigma, St. Louis, MO, USA) extravasation[18]. Briefly, Evans blue (20 mg/kg body weight) was injected into the jugular vein of rats, 30 min before duct infusion. Lung tissue samples were obtained 6 h after duct infusion, sectioned and immersed in a formamide solution, homogenized for 2 min. After incubation at room temperature for 24 h, the suspension was centrifuged at 4000 *g* for 30 min. The amount of dye extracted was determined spectrophotometrically at 620 nm and calculated from a standard curve established with a known amount of Evans blue. Results were corrected by the wet/dry lung tissue ratio and expressed as the dye content per dry weight of lung tissue (μg/g tissue).

Western blotting analysis

Western blotting analysis was performed as previously described[19]. Total protein (20 μg) was separated from each sample by electrophoresis on a 4%-20% SDS-polyacrylamide gel and electroblotted onto polyvinylidene difluoride membranes. Membranes were blocked in a blocking solution, incubated overnight with primary antibodies, and developed with a horseradish peroxidase-conjugated secondary antibody (Kangchen Biotech, Shanghai, China) diluted at 1:1000. Primary antibody (Zymed Laboratories, South San Francisco, CA, USA) was diluted as follows: claudin-4 at 1:100, claudin-5 at 1:100, and occludin at 1:300. The immune complexes were then visualized on X-ray film using chemiluminecent HRP substrate (Millipore, Boston, MA, USA). Additional immunoblots were performed using GAPDH antibody (Abcam, OFW, UK) as the primary antibody to evaluate equal loading.

Immunohistological analysis

Lung tissue sections (4 μm) were dewaxed in graded alcohols, and washed with tap water. Endogenous peroxidase activity was blocked with 3% (v/v) H_2O_2, and antigen was retrieved with microwave in 0.01 mol/L citrate buffer. The sections were then washed with phosphate-buffered saline (PBS, 0.1 mol/L). Mouse anti-rat claudin-4 and claudin-5, and rabbit anti-rat occludin polyclonal antibodies (Zymed Laboratories, South San Francisco, CA, USA) were diluted at 1:100 and incubated overnight at 4℃. The sections were washed 4 times with PBS, 5 min once. Power vision two-step histostaining reagent (ImmunoVision Technologies, Norwell, MA, USA) was used for detection of claudin and occludin expression. All sections were developed using diaminobenzidine and counterstained with hematoxylin.

Quantitative reverse transcriptase-polymerase chain reaction analysis

Total RNA was extracted with a TRIzol kit (Invitrogen Carlsbad, CA, USA) and converted to cDNA with a first strand cDNA synthesis kit (Fermentas, Burlington, Canada). Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using SYBR Green SuperMix-UDG (Invitrogen Carlsbad, CA, USA). The primer sequences used for PCR are as follows: claudin-4 (forward 5'-CCTTTCCCATACGGTCTTGCT-3', reverse 5'-CCCGTACCTTCCACAGACTG-3'), claudin-5 (forward 5'-TACTCAGCACCAAGGCGAACCAC-3', reverse 5'-GCGGCTTCCCACATCGGTC-3'), occludin (forward 5'-AGTACATGGCTGCTGCTGATG-3', reverse 5'-CCCACCATCCTCTTGATGTGT-3'), GAPDH (forward 5'-CAGTGCCAGCCTCGTCTCATA-3', reverse 5'-TGCCGTGGGTAGAGTCATA-3'). Amplification was performed at 50℃ for 2 min (UDG incubation), at 95℃ for 2 min, followed by 40 cycles of denaturing at 95℃ for 15 s and annealing at 60℃ for 30 s. All reactions were performed in triplicate. Melting curve analysis was performed to ensure the specificity of quantitative PCR. Data analysis was performed as previously described[20], with GAPDH used as a reference gene.

Statistical analysis

Data were expressed as mean ± SD. ANOVA was used to analyze differences between experimental and control groups. Student-Newman-Kleus method was used for multiple pair-wise comparisons. All statistical analyses were carried out using the SPSS version 11.5 for Windows (Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

RESULTS

Emodin attenuated pulmonary edema and inflammation in rats with acute pancreatitis

The appropriate induction of pancreatitis-associated lung injury was demonstrated by histology and elevated serum amylase activity (Figure 1 and Table 1). Lung injury was characterized by pulmonary edema, leukocyte infiltration, and alveolar collapse. Pulmonary pathological scores and serum amylase activity were significantly lower after treatment with emodin.

Pulmonary edema was evaluated by measuring the water content in lung tissue samples and expressed as wet/dry ratio, which was significantly decreased after treatment with emodin (Table 1).

In the present study, the effect of emodin on pulmonary inflammation and MPO activity was evaluated. The TNF-α and IL-6 levels and MPO activity were decreased after treatment with emodin (Table 2).

Emodin promoted expression of claudin-4, claudin-5 and occludin in rats with acute pancreatitis

The expression levels of claudin-4, claudin-5, and occludin were markedly lower in experimental group than in control group (data not shown). Immunolocalization of claudin-4, claudin-5 and occludin in lung tissue samples was investigated with immunohistochemical staining. Moderate immunostaining of claudin-4, claudin-5, and occludin was detected in control group, which was distributed in alveolar epithelium, vascular endothelium, and bronchial epithelium, respectively (Figure 2A, D and G). Immunostaining of claudin-4, claudin-5, and occludin was markedly decreased in experimental group (Figure 2B, E and H), and moderately elevated after treatment with emodin (Figure 2C, F and I).

RT-PCR analysis showed that emodin could increase the expression levels of claudin-4, claudin-5, and occludin mRNA in rats with acute pancreatitis (Figure 3A).

Western blotting analysis showed that the expression levels of claudin-4, claudin-5, and occludin were significantly higher in emodin treatment group than in pancreatitis group (Figure 3B and C).

Emodin enhanced alveolar epithelial barrier function in rats with acute pancreatitis

The pulmonary dye extravasation, as a marker of local paracellular permeability, was significantly reduced in rats with acute pancreatitis after treatment with emodin, indicating that emodin can augment alveolar epithelial barrier function (Figure 4).

Table 1 Serum amylase level and pulmonary pathological scores in different groups (mean ± SD)

Group	Amylase (U/mL)	Edema (wet/dry)	Pathological score
Sham	3.5 ± 0.4	1.7 ± 0.2	0.3 ± 0.1
Pancreatitis	13.8 ± 1.6[a]	6.4 ± 0.5[a]	7.4 ± 0.8[a]
Emodin	8.2 ± 1.1[c]	4.1 ± 0.6[c]	4.8 ± 0.7[c]

Six rats were studied in each experimental group. [a]$P < 0.05$ *vs* sham group; [c]$P < 0.05$ *vs* pancreatitis group.

Table 2 Pulmonary cytokines and MPO in different groups (mean ± SD)

Group	TNF-α (pg/mg protein)	IL-6 (pg/mg protein)	MPO (U/mg protein)
Sham	2.8 ± 0.3	25.8 ± 2.9	3.1 ± 0.3
Pancreatitis	34.1 ± 3.5[a]	114.4 ± 12.6[a]	25.8 ± 2.9[a]
Emodin	25.4 ± 2.4[c]	93.0 ± 9.8[c]	20.3 ± 1.9[c]

Six rats were studied in each experimental group. [a]$P < 0.05$ *vs* sham group; [c]$P < 0.05$ *vs* pancreatitis group. MPO: Myeloperoxidase; TNF: Tumor necrosis factor.

DISCUSSION

In the present study, we identified the down-regulation of claudin-4, claudin-5, and occludin in rats with acute pancreatitis induced by sodium taurocholate. Intravenous administration of emodin promoted the down-regulation of tight junctions, enhanced alveolar epithelial barrier function, attenuated pulmonary edema and inflammatory infiltration in rats with acute pancreatitis.

Among the systemic complications of severe acute pancreatitis, pulmonary complication, also known as pancreatitis-associated lung injury, is the most frequent and serious[6]. Pancreatitis-associated lung injury is characterized by significant pulmonary edema, hyperemia and inflammatory infiltration in alveoli[5]. Increased interstitial edema cuts down the transport of carbon dioxide through the alveolar barrier, causing respiratory distress syndrome. It has been recently reported that claudins, the key components of tight junctions, restrict paracellular movement of water, proteins, and solutes across cellular barriers including pulmonary vascular endothelium and alveolar epithelium[8]. Disruption of claudins impairs barrier function and increases paracellular permeability, which may allow noxious contents to enter pulmonary interstitium and alveoli, further aggravating pulmonary edema and inflammation[5-9].

Recently, several studies have demonstrated the localization and function of claudin-4 in pulmonary cellular barriers[9-11]. In human airway epithelia, elevated claudin-4 level is associated with increased transepithelial electrical resistance, indicating that claudin-4 plays a role in alveolar epithelial barrier function[10]. Although increased claudin-4 expression has been found in a mice model of acute lung injury, inhibition of claudin-4 can lead to pulmonary edema in mice by decreasing transepithelial electrical

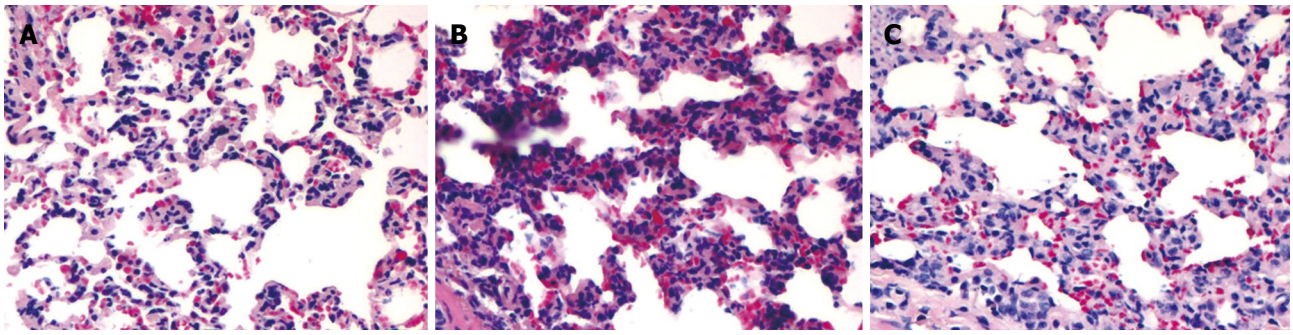


Figure 1 Effects of emodin on pancreatitis-associated lung injury in sham operation group (A), pancreatitis group (B), and emodin group (C) (original magnification, × 200).

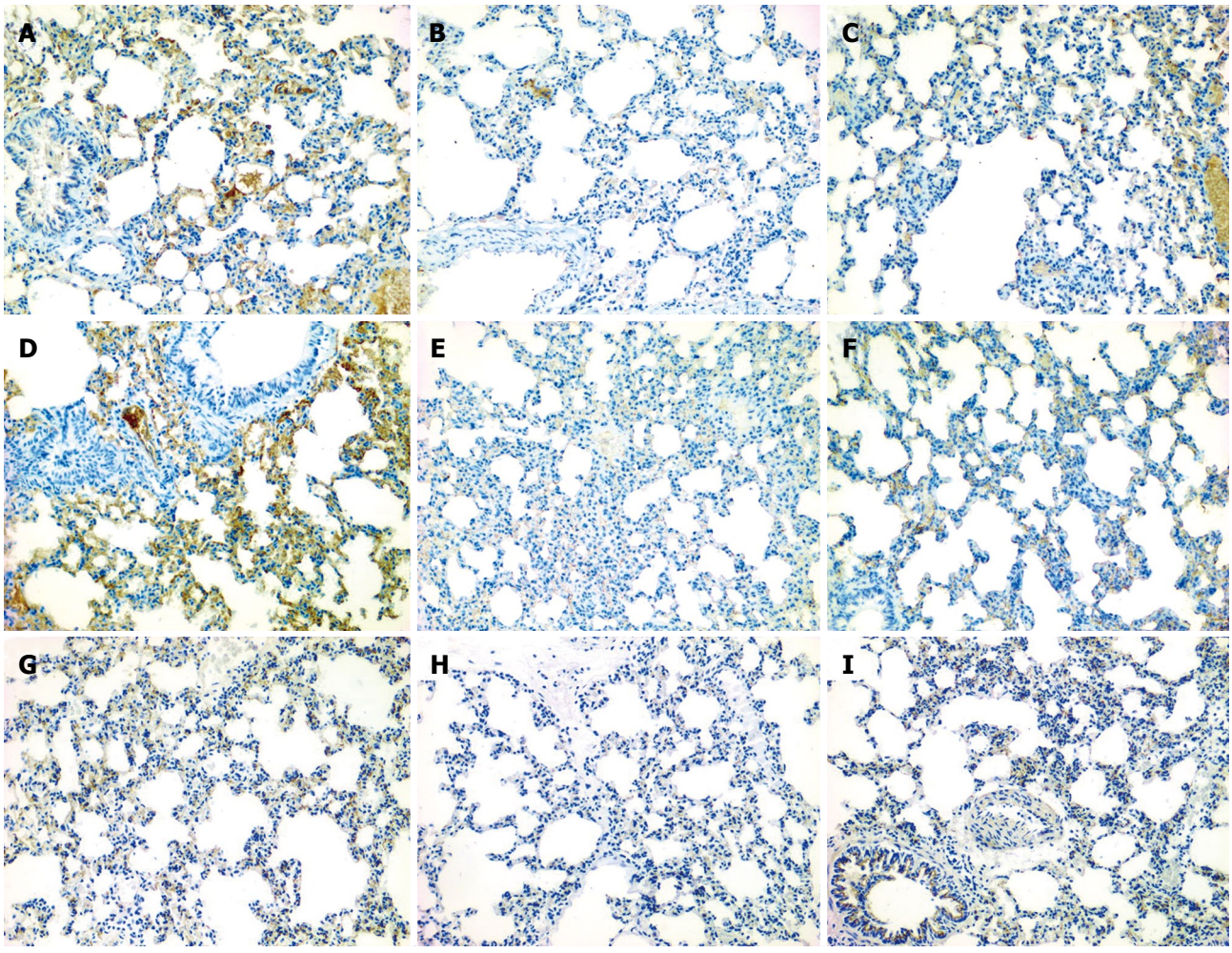


Figure 2 Immunohistochemical staining of claudin-4 (A-C), claudin-5 (D-F) and occludin (G-I) in sham operation group (A, D, G), pancreatitis group (B, E, H), and emodin group (C, F, I) (original magnification, × 200).

resistance and air space fluid clearance, suggesting that claudin-4 plays an important role in alveolar epithelial barrier function, and early increased claudin-4 expression may represent a mechanism by which pulmonary edema is limited[9]. Similar to claudin-4, claudin-5 also plays a role in cellular barrier function. Recombinant claudin-5 protects brain microvascular endothelial cell cultures against increased paracellular permeability induced by VEGF, showing that claudin-5 is a key determinant of blood-brain barrier function[21]. It has been recently reported that expression of pulmonary claudin-5 is decreased in models of carrageenan-induced acute lung inflammation, associated with the decreased pulmonary paracellular permeability, suggesting that claudin-5 may play role in alveolar epithelial barrier function[11].

Occludin shares a very similar membrane location with claudin. Based on the staining feature of claudins and occludin along the endothelial cell borders, Persid-

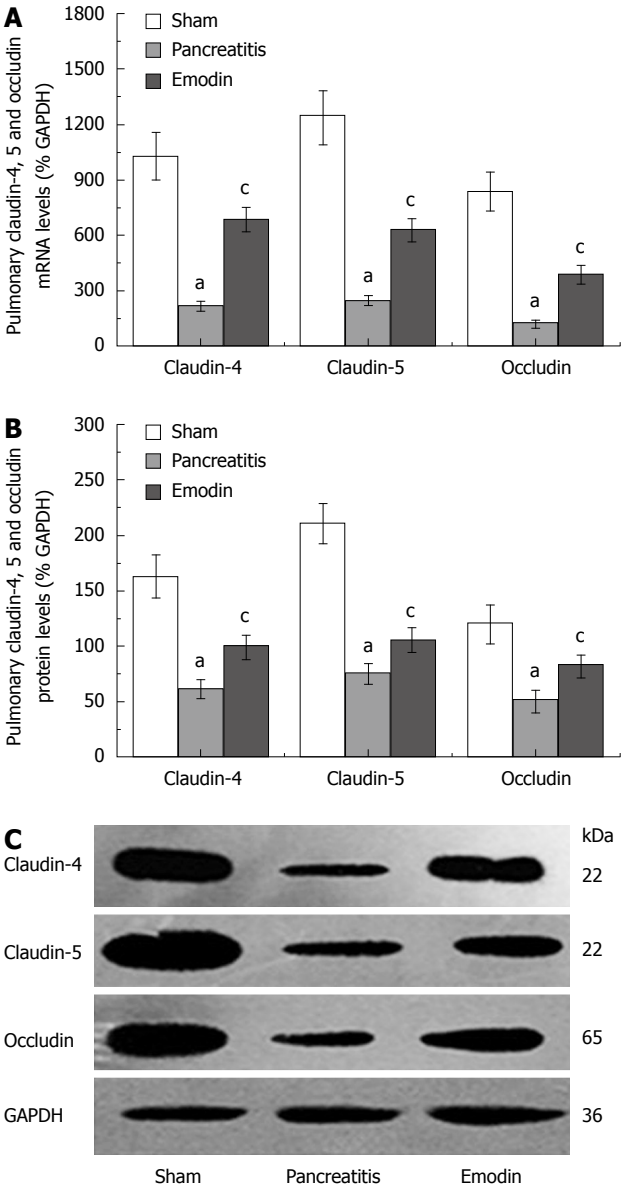


Figure 3 Effects of emodin on claudin-4 (A), claudin-5 (B), and occludin (C) mRNA transcription and protein synthesis in rats. Six rats were studied in each experimental group. Results are expressed as mean ± SD. $^{a}P < 0.05$ *vs* sham group; $^{c}P < 0.05$ *vs* pancreatitis group.

sky *et al*[22] speculated that claudins form the primary "makeup" of the tight junctions, and occludin further enhances tight junction tightness. In ethanol abused rats, which is decreased mRNA and protein expression of occludin has also been observed in lung tissues, associated with increased bronchoalveolar epithelial permeability[12]. Azithromycin-induced processing of occludin is accompanied by increased transepithelial electrical resistance[10], suggesting that occludin alteration may be related with alveolar barrier function.

In the present study, we identified the localization of claudin-4, claudin-5, and occludin in lung tissue samples from rats with acute pancreatitis, and found that claudin-4 and claudin-5 were uniformly and continuously distributed along the alveolar epithelium and vascular endothelium in normal lung tissue samples, which are consistent with the reported findings[9-11]. Furthermore, occludin was uniformly and continuously distributed along the alveolar epithelium, vascular endothelium, and bronchiolar epithelium, which is in line with the reported results[10,12]. In this study, RT-PCR and Western blotting showed that the expression of claudin-4, claudin-5 and occludin was down-regulated in lung tissue samples from rats with acute pancreatitis. Aggravated pulmonary edema and increased paracellular permeability (marked by extravasation of Evans blue) were in parallel with the down-regulation of claudin-4, claudin-5 and occludin expression, which is consistent with the findings in previous studies[9-12], suggesting that claudin-4, claudin-5 and occludin may play a role in alveolar barrier function.

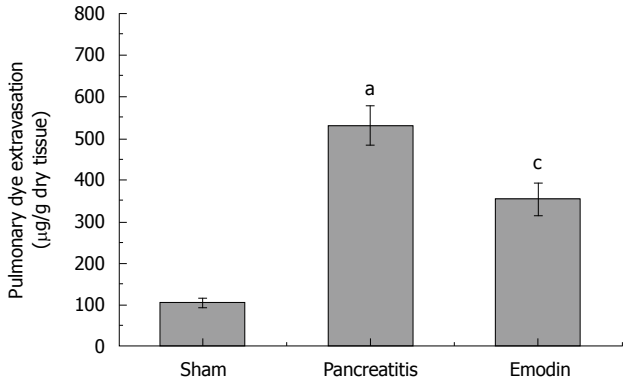


Figure 4 Effects of emodin on alveolar epithelial barrier in rats. Six rats were studied in each experimental group. Results are expressed as mean ± SD. $^{a}P < 0.05$ *vs* sham group; $^{c}P < 0.05$ *vs* pancreatitis group.

In the present study, emodin significantly promoted the expression of claudin-4, claudin-5 and occludin at mRNA transcription and protein synthesis level, and decreased pulmonary edema and paracellular permeability. Based on the previous and present studies, we speculate that emodin may contribute, in part at least, to the expression of claudin-4, claudin-5 and occludin by increasing the alveolar barrier function.

Emodin has long been used for anti-inflammatory purposes. Many studies have demonstrated that emodin intervention can significantly decrease TNF-α and IL-6 levels, or MPO activity in lung tissues[13,23,24], and the mechanism of emodin underlying cytokine inhibition is involved in NF-κB activity suppression[23-26]. Moreover, emodin also has antioxidant effects, promotes generation of ATP and antioxidant components, such as glutathione, α-tocopherol, and superoxide dismutase[27], and exhibits a promising free radical scavenging activity[28]. It has been shown that emodin markedly reduces serum amylase, TNF-α and IL-6 levels, attenuates lung damage in rats with acute pancreatitis[14,29,30], which is in line with the present study. Considering that MPO activity is a marker of local leukocyte sequestration[30], the results of our present study suggest that emodin ameliorates pancreatitis-associated lung injury by inhibiting the production of cytokines and the infiltration of leukocytes in lungs.

In conclusion, emodin can attenuate pulmonary edema and inflammation, enhance alveolar epithelial barrier function, and promote expression of claudin-4, claudin-5 and occludin in lung tissues.

COMMENTS

Background

The mortality associated with acute pancreatitis is attributed to inflammation-related complications such as pancreatitis-associated lung injury. Interventions with emodin are likely to reduce its morbidity and mortality of the disease.

Research frontiers

Claudins and occludin, the key components of tight junctions, restrict the paracellular movement of water, proteins, and solutes across cellular barriers including alveolar epithelium. Emodin, an anthraquinone derivative from the Chinese herb *Radix et Rhizoma Rhei*, has been used for anti-inflammatory purposes. Weather emodin has effects on pulmonary tight junction expression and alveolar epithelial barrier has not been defined.

Innovations and breakthroughs

A recent report has highlighted the importance of tight junction components in a model of ethanol-induced lung injury, or carrageenan-induced acute lung inflammation. This is the first study to report that down-regulation of pulmonary claudin-4, claudin-5 and occludin is parallel with pancreatitis-associated lung injury. Emodin can promote expression of claudin-4, claudin-5 and occluding in lungs, enhance alveolar epithelial barrier, and inhibit pulmonary inflammation.

Applications

The results of this study may improve our understanding of the pathogenesis of pancreatitis-associated lung injury. The present study also provides evidence for emodin in treatment of lung injury.

Peer review

This is a largely observational study representing an incremental advance in treatment of acute pancreatitis with emodin.

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ORIGINAL ARTICLE

Sulforaphane protects liver injury induced by intestinal ischemia reperfusion through Nrf2-ARE pathway

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Abstract

AIM: To investigate the effect of sulforaphane (SFN) on regulation of NF-E2-related factor-2 (Nrf2)-antioxidant response element (ARE) pathway in liver injury induced by intestinal ischemia/reperfusion (I/R).

METHODS: Rats were divided randomly into four experimental groups: control, SFN control, intestinal I/R and SFN pretreatment groups (n = 8 in each group). The intestinal I/R model was established by clamping the superior mesenteric artery for 1 h and 2 h reperfusion. In the SFN pretreatment group, surgery was performed as in the intestinal I/R group, with intraperitoneal administration of 3 mg/kg SFN 1 h before the operation. Intestine and liver histology was investigated. Serum levels of aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were measured. Liver tissue superoxide dismutase (SOD), myeloperoxidase (MPO), glutathione (GSH) and glutathione peroxidase (GSH-Px) activity were assayed. The liver transcription factor Nrf2 and heme oxygenase-1 (HO-1) were determined by immunohistochemical analysis and Western blotting analysis.

RESULTS: Intestinal I/R induced intestinal and liver injury, characterized by histological changes as well as a significant increase in serum AST and ALT levels (AST: 260.13 ± 40.17 U/L *vs* 186.00 ± 24.21 U/L, $P < 0.01$; ALT: 139.63 ± 11.35 U/L *vs* 48.38 ± 10.73 U/L, $P < 0.01$), all of which were reduced by pretreatment with SFN, respectively (AST: 260.13 ± 40.17 U/L *vs* 216.63 ± 22.65 U/L, $P < 0.05$; ALT: 139.63 ± 11.35 U/L *vs* 97.63 ± 15.56 U/L, $P < 0.01$). The activity of SOD in the liver tissue decreased after intestinal I/R ($P < 0.01$), which was enhanced by SFN pretreatment ($P < 0.05$). In addition, compared with the control group, SFN markedly reduced liver tissue MPO activity ($P < 0.05$) and elevated liver tissue GSH and GSH-Px activity ($P < 0.05$, $P < 0.05$), which was in parallel with the increased level of liver Nrf2 and HO-1 expression.

CONCLUSION: SFN pretreatment attenuates liver injury induced by intestinal I/R in rats, attributable to the antioxidant effect through Nrf2-ARE pathway.

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Key words: Sulforaphane; Liver injury; Intestinal ischemia reperfusion; NF-E2-related factor-2; Antioxidant response element

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INTRODUCTION

Intestinal ischemia/reperfusion (I/R) is considered to be a grave and triggering event in development of local and distant organ dysfunction[1-4], which occurs in many clinical settings including acute mesenteric ischemia, hemorrhagic, traumatic or septic shock, severe burns, resuscitation, small bowel transplantation and thoracoabdominal aortic aneurysm repair[5,6]. The mechanism of liver injury induced by intestinal I/R is complicated, and it has not been fully elucidated although many studies have been done to mimic the pathophysiologic process by both cell culture and animal models in the past a few years[7-10].

Traditionally, decreased basement membrane integrity and barrier function of the intestine, which facilitate bacterial translocation and local production of inflammatory cytokines[11], promotes the systemic inflammatory response syndrome and multiple organ dysfunction syndrome[12,13]. Recent studies showed that reactive oxygen species (ROS) generated during tissue reperfusion, play an important role in intestinal I/R injury[14], which can initiate lipid peroxidation, oxidize proteins to inactive states and cause DNA strand breaks, and initiation of apoptotic and necrotic cascades[15]. Additionally, ROS also has a function as a second messenger to modulate the secretion of pro-inflammation cytokines and chemokines that can destroy the intestinal barrier, which is a critical junction point to amplify the inflammation reaction described above[11-13]. The administration of antioxidants has been shown to exert beneficial effects in the prevention of ischemia-reperfusion injury[16].

Sulforaphane (SFN) is a natural product derived from isothiocyanate which is present in cruciferous vegetables such as broccoli that has been used as a chemopreventive compound[17]. The cytoprotective effect of this compound is mediated by transcription factor NF-E2-related factor-2 (Nrf2), which binds to the antioxidant response element (ARE) in the promoter region of a number of genes, encoding for antioxidative and phase 2 enzymes, including heme oxygenase-1 (HO-1), NAD(P)H: quinoine oxidoreductase 1, glutathione reductase, and glutathione peroxidase (GSH-Px)[18-20]. Phase 2 enzymes play a major role in the detoxification of ROS during ischemia/reperfusion[21,22]. Nrf2 is held in the cytoplasm by a cytoskeletal-associated specific inhibitory protein (Kelch-like ECH associated protein 1, Keap1) under circumstances of normal cellular quiescent state. Upon stimulation of oxidative stress, cysteine residues within the hinge region of Keap1 can be modified and cause a conformational change in KEAP1 with the loss of Nrf2 binding, then Nrf2 translocated into the nucleus, where it heterodimerizes with members of the maf protein family, and coordinates upregulation of cytoprotective genes[20]. SFN can disrupt the Nrf2-Keap1 complex, and permit Nrf2 to translocate into the nucleus to activate the ARE-driven genes[23,24]. Studies suggest that Nrf2 activation by SFN results in effective protection from cancers[25] and renal I/R[26] by upregulating ARE-related detoxification enzymes. These studies have focused on the chemoprevention by SFN, and to our knowledge, no one has evaluated SFN to determine if it can protect liver injury induced by intestinal I/R.

In this study, we investigated the effect of SFN on liver injury following intestinal I/R and explored the mechanism of its protective action through Nrf2-ARE pathway.

MATERIALS AND METHODS

Animal and experimental design

Male Sprague-Dawley rats weighing 190-220 g (from the Animal center of Dalian Medical University, Dalian, China) were used in this study, which was approved by the Institutional Ethics Committee. All rats were provided with standard laboratory chow and water and were housed in accordance with institutional animal care policies.

The rats were divided into four experimental groups randomly: control (A), SFN control (B), intestinal I/R (C) and SFN pretreatment group (D) (n = 8 in each group). The rats in the control group underwent surgical preparation including isolation of the superior mesenteric artery (SMA) without occlusion; the rats in the SFN control group underwent surgery as in the control group with intraperitoneal administration of 3 mg/kg SFN 1 h before the operation (SFN was purchased from Sigma Chemical Company and was dissolved in 10% dimethylsulfoxide before administration); the rats in intestinal I/R group were subjected to 1 h intestinal ischemia and 2 h reperfusion after the SMA was isolated and occluded[27]; the rats in the SFN pretreatment group underwent surgery as in the intestinal I/R group with intraperitoneal administration of 3 mg/kg SFN 1 h before the operation.

The dose of SFN administration was determined according to the previous studies with modification from preliminary experiments. The rats in the control and intestinal I/R groups were treated with an equal volume of 10% dimethylsulfoxide. Two hours after reperfusion, blood, intestine and liver tissue samples were obtained for further analysis.

Intestine and liver morphological analysis

The isolated intestine and liver tissues were harvested and fixed in 10% formalin. After being embedded in paraffin, the tissues were stained with hematoxylin and eosin for light microscopy.

Measurement of serum aspartate aminotransferase and alanine aminotransferase

The serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured with an OLYMPUS AU1000 automatic analyzer (AusBio Laboratories Co., Ltd. Beijing, China).

Liver superoxide dismutase, myeloperoxidase, GSH and GSH-Px activity assay

The liver tissues were harvested and homogenized im-

mediately on ice in 5 volumes of normal saline. The homogenates were centrifuged at 1200 r/min for 10 min to remove debris. The superoxide dismutase (SOD), myeloperoxidase (MPO), GSH and GSH-Px activity in the supernatant were determined using an assay kit (Nanjing Jiancheng Corp., China), following the manufacturer's recommendations.

The SOD activity was determined by hydroxylamine assay developed from xanthine oxidase assay. One unit of SOD sample inhibited the reaction by approximately 50% of the initially measured xanthine oxidase reaction at 37℃. SOD activity in the liver tissues was expressed as units per milligram protein (U/mgprot).

One unit of MPO activity is defined as degrading 1 μmol of hydrogen peroxide at 37℃ and MPO activity of tissue was expressed as units per gram (U/g).

Concentrations of GSH were determined by the 5,5' dithio-bis 2-nitrobenzoic acid (DTNB)-GSSG reductase-recycling assay. The amount of GSH was expressed as milligrams per gram protein (mg/gprot).

GSH-Px was measured by the enzymatic reaction which was initiated by addition of H_2O_2 to the reaction mixture containing reduced glutathione, NADPH and glutathione reductase, and the change in the absorbance at 340 nm was monitored by spectrophotometer. Activity was given in units per milligram protein (U/mgprot).

Liver Nrf2 and HO-1 immunohistochemical analysis

Paraffin-embedded tissue sections, 4 μm in thickness, were stained with SP immunohistochemical technique for Nrf2 and HO-1. The immunohistochemical experiments were performed according to the manufacturer's recommendations. After being dewaxed or washed in PBS, tissue sections were cultured in 3% hydrogen peroxide to eliminate intrinsic peroxidase, and quenched in normal goat serum for 30 min. The sections were incubated overnight at 4℃ with polyclonal rabbit anti-rat Nrf2 antibody (Santa Cruz Corp., Ltd.), followed by the addition of the anti-rabbit immunoglobulin and streptavidin conjugated to horseradish peroxides. Finally, 3,3'-diaminobenzidine was used for color development, and hematoxylin was used for counter staining. The brown or dark brown staining in cytoplasm and/or nucleus was considered to be positive. The results were evaluated semi-quantitatively according to percentage of positive cells in five fields at a 400 multiple signal magnification. The protein expression in tissue sections was graded as 0: less than 5%; 1: from 6% to 25%; 2: from 26% to 50%; 3: from 51% to 75%; and 4: more than 75%[28].

Western blotting analysis of liver Nrf2 and HO-1

Nrf2 is a nuclear transcriptional factor that binds to the ARE in the promoter region of a number of genes, encoding for antioxidative and phase 2 enzymes such as HO-1 and GSH-Px. The level of Nrf2 in the nucleus was examined by Western blotting to assess Nrf2 activation.

Cellular plasma and nuclear protein were extracted from frozen liver tissues with a protein extraction kit (Pierce, Meridian Road, Rockford, IL, USA) for HO-1 and Nrf2 measurement separately. The protein was separated by 10% SDS-PAGE gel electrophoresis. The protein was electroblotted onto NC membranes (Millipore, Bedford, MA, USA) at 9 V for 30 min. The transferred membranes were then incubated overnight at 4℃ with rabbit polyclonal antibody HO-1, Nrf2, GAPDH and PCNA (Santa Cruz Corp., Ltd.) against rat in TBS-T (10 mmol/L Tris-HC1, pH 7.5, 150 mmol/L NaC1, 0.1% Tween-20) containing 5% skim milk. After washing three times in TBS-T, the membranes were incubated for 1 h at 37℃ with an anti-rabbit IgG conjugated with horseradish peroxidase. The signals were visualized using the DAB assay kit (Fuzhou Maixin Biological Technology Co., Ltd, Fuzhou, China) and documented with a gel imaging system (UVP Bioimaging System). The signals were analyzed with software Gel-Pro Analyzer 4.0.

Statistical analysis

All data, which expressed as the mean ± SD, were compared using the paired Student's *t* test with the SigmaStat 3.5 statistical software package. One-way analysis of variance (ANOVA) was used to determine significant differences in antioxidant enzyme activities between the groups. A value of $P < 0.05$ was considered significant.

RESULTS

Changes of liver histology and serum AST and ALT levels

Intestinal I/R induced apparent intestine and liver injury at 2 h after reperfusion, manifested as histological changes in the intestine and liver with edema, hemorrhage and neutrophil infiltration (Figure 1), as well as a significant increase in serum AST and ALT level (AST: 260.13 ± 40.17 U/L *vs* 186.00 ± 24.21 U/L, $P < 0.01$; ALT: 139.63 ± 11.35 U/L *vs* 48.38 ± 10.73 U/L, $P < 0.01$, Figure 2) when compared with the control group. Compared with the intestinal I/R group, the intestinal and liver pathological damage was improved in the SFN pretreatment group. In addition, there was a significant difference in the liver function between the intestinal I/R and SFN pretreatment group in serum AST and ALT levels (AST: 260.13 ± 40.17 U/L *vs* 216.63 ± 22.65 U/L, $P < 0.05$; ALT: 139.63 ± 11.35 U/L *vs* 97.63 ± 15.56 U/L, $P < 0.01$, Figure 2), which indicates that SFN significantly attenuated the intestinal I/R-induced liver injury. There was no significant difference between the SFN control group and the control group in liver pathological damage (Figure 1) and serum AST (AST: 193.38 ± 34.63 U/L *vs* 186.00 ± 24.21 U/L, $P > 0.05$, Figure 2) and ALT (ALT: 48.00 ± 8.52 U/L *vs* 48.38 ± 10.73 U/L, $P > 0.05$, Figure 2).

Changes of liver SOD and MPO activity

SOD is the major enzyme for scavenging ROS, and its activity can reflect its functional status. The liver homogenate SOD activity in the intestinal I/R group decreased significantly at 2 h of reperfusion in comparison with the control group ($P < 0.01$, Figure 3). SOD activity was elevated markedly after SFN pretreatment compared

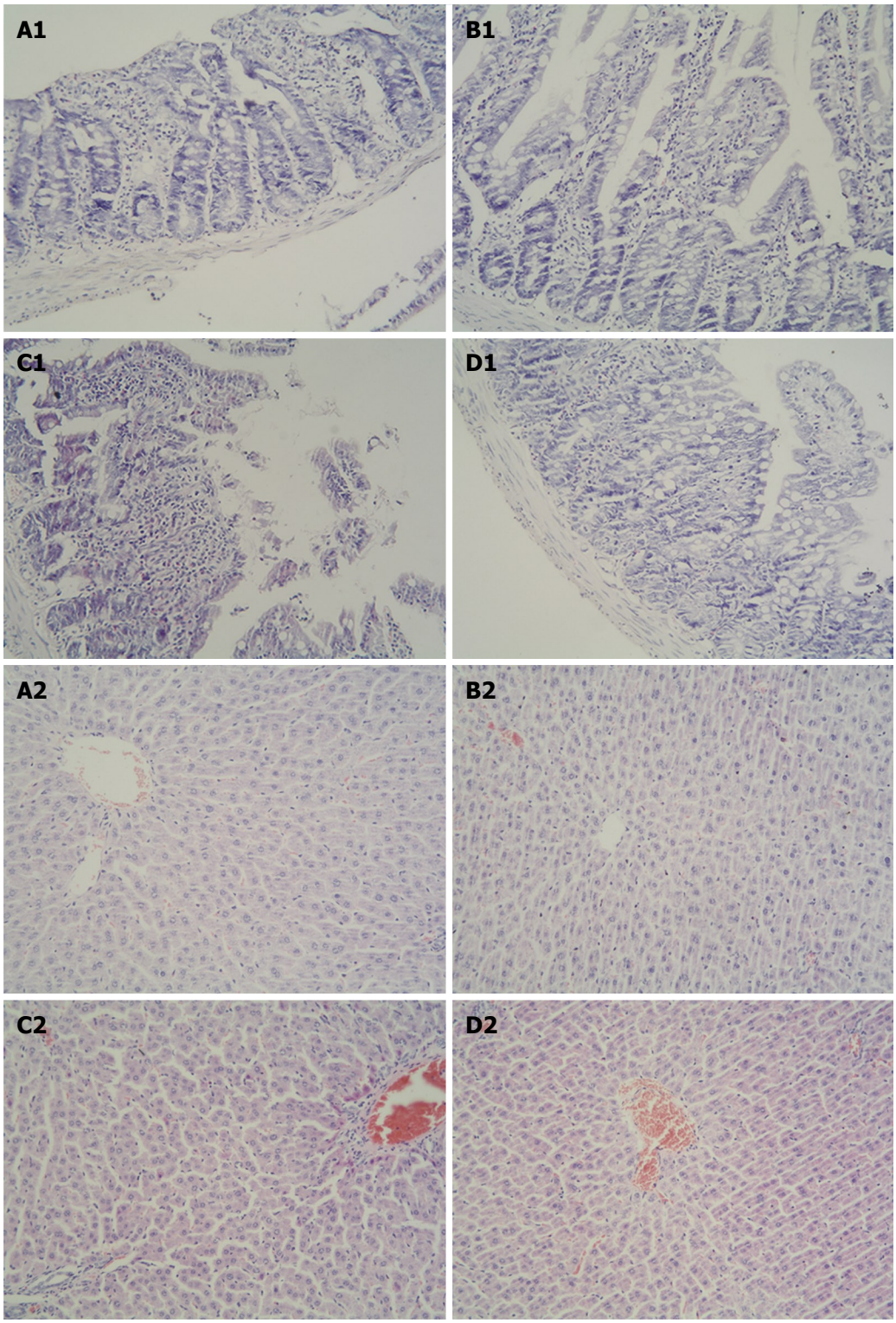


Figure 1 Changes in histology of intestine (A1-D1) and liver (A2-D2) tissues (HE stain, × 200) in the control (A), sulforaphane (SFN) control (B), intestinal ischemia/reperfusion (I/R) (C) (1 h ischemia and 2 h reperfusion) and SFN pretreatment (D) groups. A, B: Normal structure of intestine and liver; C: Edema, hemorrhage and neutrophil infiltration were observed in intestinal mucosa and liver tissue; D: Relatively normal histology of intestine and liver with less inflammatory cell infiltration.

with the intestinal I/R group ($P < 0.05$, Figure 3).

MPO activity in the liver homogenate was measured for estimating the leukocyte recruitment to liver tissues. The liver tissue MPO activity increased significantly after intestinal I/R at 2 h of reperfusion compared with the control group ($P < 0.05$, Figure 3). The administration of SFN reduced the MPO activity in liver tissues significantly in comparison with the intestinal I/R group ($P < 0.05$, Figure 3), thus indicating that SFN alleviated the leukocyte recruitment to liver tissues.

GSH is a kind of antioxidant which can scavenge ROS. Phase 2 enzyme GSH-Px present in the cell can

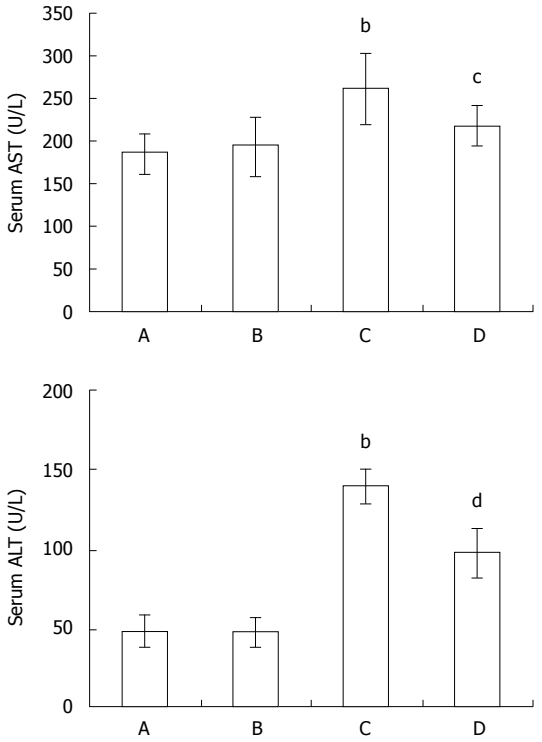


Figure 2 Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) (mean ± SD, U/L) levels in the control (A), SFN control (B), intestinal I/R (C) (1 h ischemia and 2 h reperfusion) and SFN pretreatment (D) groups. $^{b}P < 0.01$ *vs* control group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* intestinal I/R group.

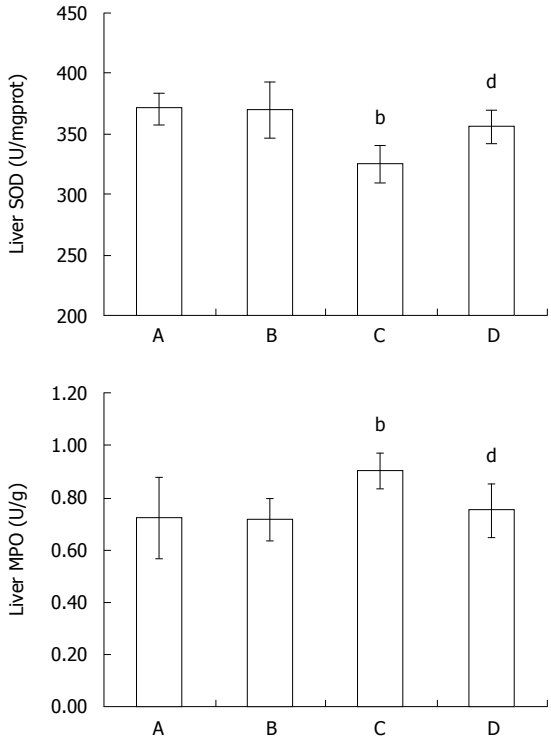


Figure 3 Liver tissue superoxide dismutase (SOD) (mean ± SD, U/mgprot) and myeloperoxidase (MPO) (mean ± SD, U/g) level in the control (A), SFN control (B), intestinal I/R (C) (1 h ischemia and 2 h reperfusion) and SFN pretreatment (D) groups. $^{b}P < 0.01$ *vs* control group; $^{d}P < 0.01$ *vs* intestinal I/R group.

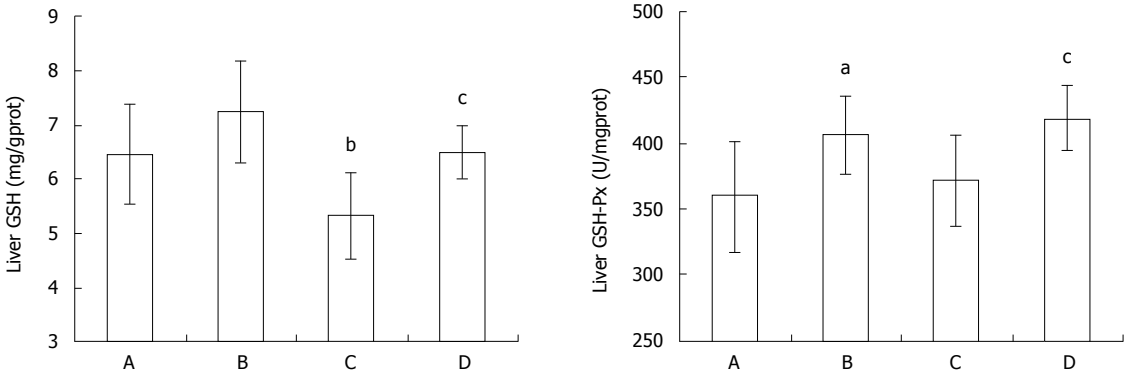


Figure 4 Liver tissue glutathione (GSH) and glutathione peroxidase (GSH-Px) (mean ± SD, U/gprot) level in the control (A), SFN control (B), intestinal I/R (C) (1 h ischemia and 2 h reperfusion) and SFN pretreatment (D) groups. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group; $^{c}P < 0.05$ *vs* intestinal I/R group.

catalyze this reaction. The liver homogenate GSH activity in the intestinal I/R group decreased significantly at 2h of reperfusion as compared with the control group ($P < 0.01$, Figure 4). GSH activity was elevated markedly after SFN pretreatment in comparison with the intestinal I/R group ($P < 0.05$, Figure 4).

The increased activity of the liver tissue GSH-Px was significant after SFN pretreatment as against the control group ($P < 0.05$, Figure 4). GSH-Px activity was elevated markedly after SFN pretreatment in comparison with the intestinal I/R group ($P < 0.05$, Figure 4), thus supporting the hepatoprotective effect of SFN.

Immunohistochemical expression of liver Nrf2 and HO-1

The expression of Nrf2 in control group showed light brown immunostaining in cytoplasm and no staining in the nuclei. The significantly positive expressions of Nrf2 as strong brown staining in cytoplasm and nuclei were observed in the intestinal I/R group and SFN control groups ($P < 0.01$, $P < 0.01$, Figures 5 and 6). Compared with the intestinal I/R group, the positive rates of Nrf2 expression increased significantly in SFN pretreatment group ($P < 0.01$, Figures 5 and 6).

The expression of HO-1 in the control group showed little brown immunostaining in cytoplasm while signifi-

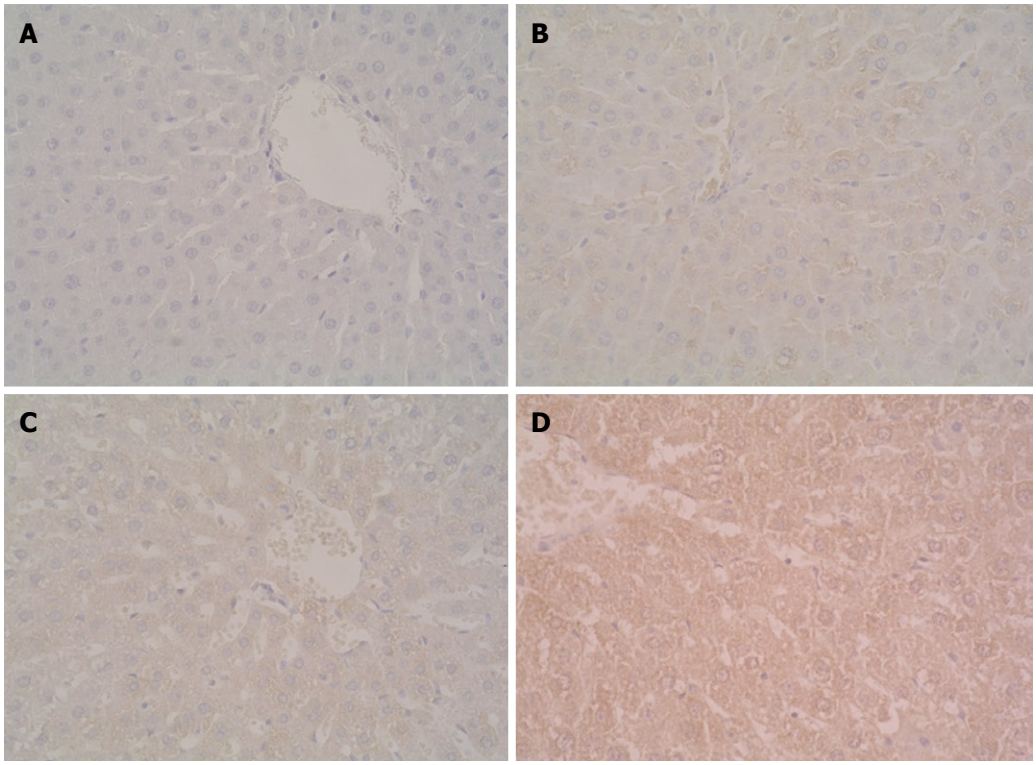


Figure 5 Immunohistochemical staining of liver NF-E2-related factor-2 (Nrf2) expression (original magnification × 400) in the control (A), SFN control (B), intestinal I/R (C) (1 h ischemia and 2 h reperfusion) and SFN pretreatment (D) groups.

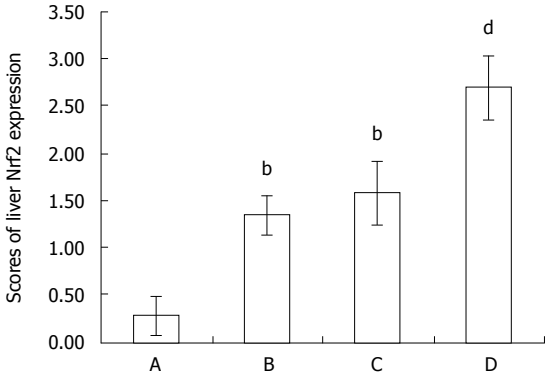


Figure 6 Immunohistochemical results (semi-quantitative analysis) of liver Nrf2 expression in the control (A), SFN control (B), intestinal I/R (C) (1 h ischemia and 2 h reperfusion) and SFN pretreatment (D) groups. Data are presented as mean ± SD of eight animals. $^{b}P < 0.01$ *vs* control group; $^{d}P < 0.01$ *vs* intestinal I/R group.

cant positive expression of HO-1 as brown staining in cytoplasm was observed in the intestinal I/R group and SFN control groups ($P < 0.01$, $P < 0.01$, Figures 7 and 8). Compared with the intestinal I/R group, the positive rates of HO-1 expression increased significantly in cytoplasm in SFN pretreatment group ($P < 0.01$, Figures 7 and 8).

Western blotting analysis of liver Nrf2 and HO-1 expression

The expression of nuclear Nrf2 of the liver tissues in animals with 2-h reperfusion is shown in Figure 9. The results showed a weak Nrf2 positive signal in the liver of the control group. However, a strong Nrf2 protein signal was found in the intestinal I/R group ($P < 0.01$, Figure 9). The signal enhanced significantly in the SFN pretreatment group in comparison with the intestinal I/R group ($P < 0.01$, Figure 9), thereby indicating that SFN could increase the Nrf2 activation in liver tissues.

Western blotting showed weak positive staining for HO-1 in the liver in the control group. However, a significant HO-1 signal was observed in the intestinal I/R group. Compared with the control group, IOD level of HO-1 increased markedly ($P < 0.01$, Figure 9). The signal enhanced significantly in the SFN pretreatment group in comparison with the intestinal I/R group ($P < 0.01$, Figure 9), hinting that HO-1 was activated in liver tissues caused by SFN.

DISCUSSION

Intestinal I/R is not only necessary to the intestine itself, but involves severe distant organ dysfunction. The liver is the first distant organ involved in the severe attack from intestinal I/R[8] due to its vasculature being coupled in series with that of the intestine[29]. During the reperfusion, ROS formed as participants in the I/R-induced leukocyte-mediated liver inflammation responses[30-32], and many clinical and experimental studies showed that ROS plays a crucial role in the pathogenesis of I/R[7,33].

ROS can be restrained by endogenous free radical scavengers such as SOD, catalase, the glutathione peroxidase/glutathione/glutathione reductase system and the thioredoxin peroxidase/thioredoxin/thioredoxin reduc-

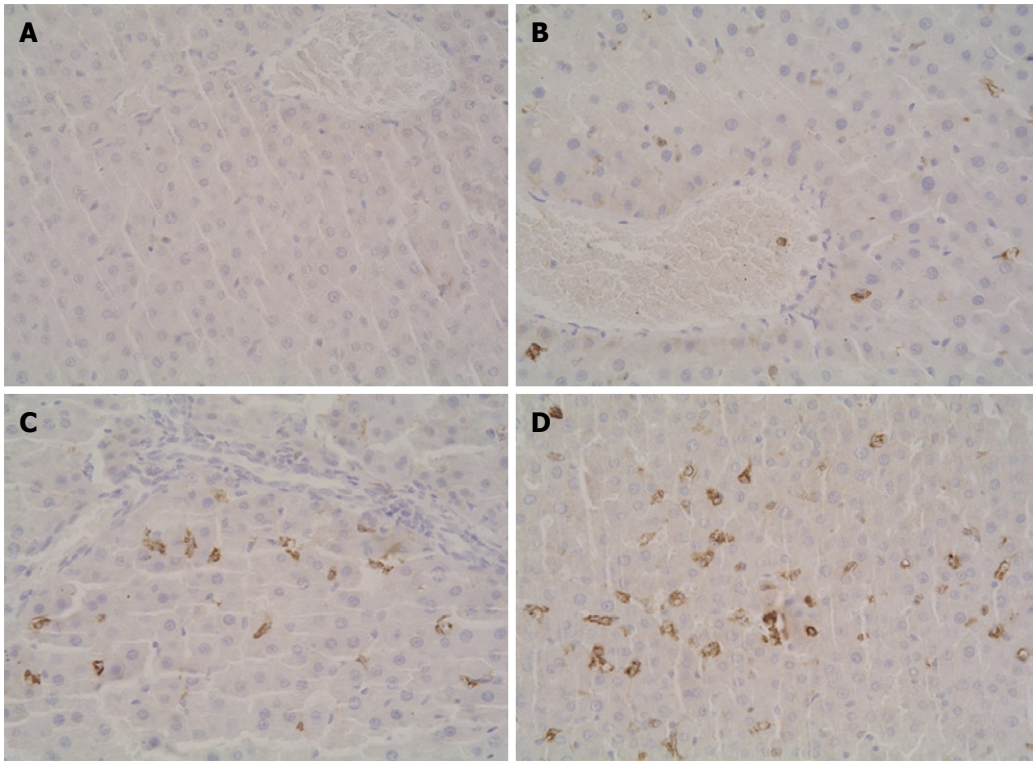


Figure 7 Immunohistochemical staining of liver heme oxygenase-1 (HO-1) expression (original magnification × 400) in the control (A), SFN control (B), intestinal I/R (C) (1 h ischemia and 2 h reperfusion) and SFN pretreatment (D) groups.

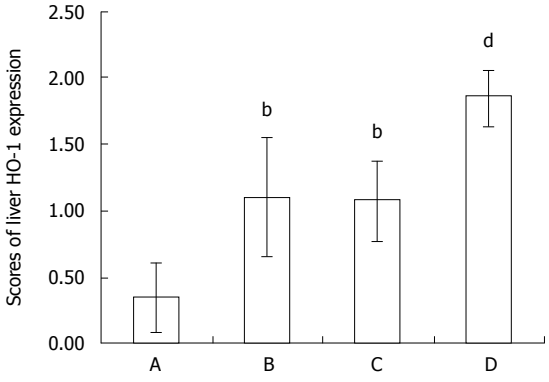


Figure 8 Immunohistochemical results (semi-quantitative analysis) of liver HO-1 expression in the control (A), SFN control (B), intestinal I/R (C) (1 h ischemia and 2 h reperfusion) and SFN pretreatment (D) groups. Data are presented as mean ± SD of eight animals. $^{b}P < 0.01$ *vs* control group; $^{d}P < 0.01$ *vs* intestinal I/R group.

tase system[34]. SOD catalyses the dismutation of the superoxide anion ($O_2^{\cdot}$) into H_2O_2, which can be transformed into H_2O and O_2 by catalase (CAT). GSH as a kind of endogenous free radical scavenger can be regulated by GSH-Px. Ros also oxidizes proteins, induces lipid peroxidation and initiates DNA stand breaks, which can be blocked by the above-mentioned antioxidative agents.

ARE is an enhancer element that initiates the transcription of a battery of genes encoding phase 2 enzymes[35]. Activation of gene transcription through the ARE is mediated primarily by Nrf2[36]. Under basal conditions, Nrf2 is sequestered in the cytoplasm by Keap1, and upon exposure of cells to inducers such as oxidative stress and certain chemopreventive agents, Nrf2 dissociates from Keap1, translocates to the nucleus, binds to ARE, and transactivates phase 2 detoxifying and antioxidant genes[37]. Recent studies have implied that the activation of Nrf2/ARE pathway attenuate ischemia/reperfusion injury of the heart, brain and kidney[26,38,39]. And SFN induces Nrf2-driven phase 2 enzyme expression by modulating the activation in kidney ischemia reperfusion injury[40].

In this study, we evaluated the effect of SFN on regulating Nrf2/ARE in liver injury of the animal intestinal I/R model. We found that Nrf2 activation by sulforaphane pretreatment protected liver against injury induced by intestinal I/R, which was characterized by improved alternation in liver tissue pathology and liver function, and an enhanced antioxidant capacity, being consistent with the Nrf2 expression and content changes.

GSH-Px and HO-1 are two kinds of phase 2 enzymes. HO-1 is a ubiquitous heat shock protein (HSP32) that is highly induced by diverse stress-related conditions[41]. It is upregulated in response to oxidative stress in many tissues, providing generalized endogenous protection against oxidative stress[42]. Our results indicated that GSH-Px and HO-1 can be upregulated by SFN pretreatment through Nrf2-ARE pathway. Therefore, development of a kind of strategy to reduce oxidative stress by inducing endogenous phase 2 enzymes is attractive, which can be regulated by Nrf2. In order to clarify the mechanism comprehensively, further researches should determine the indexes of cell death such as apoptosis and inflammation in this pathophysiology process.

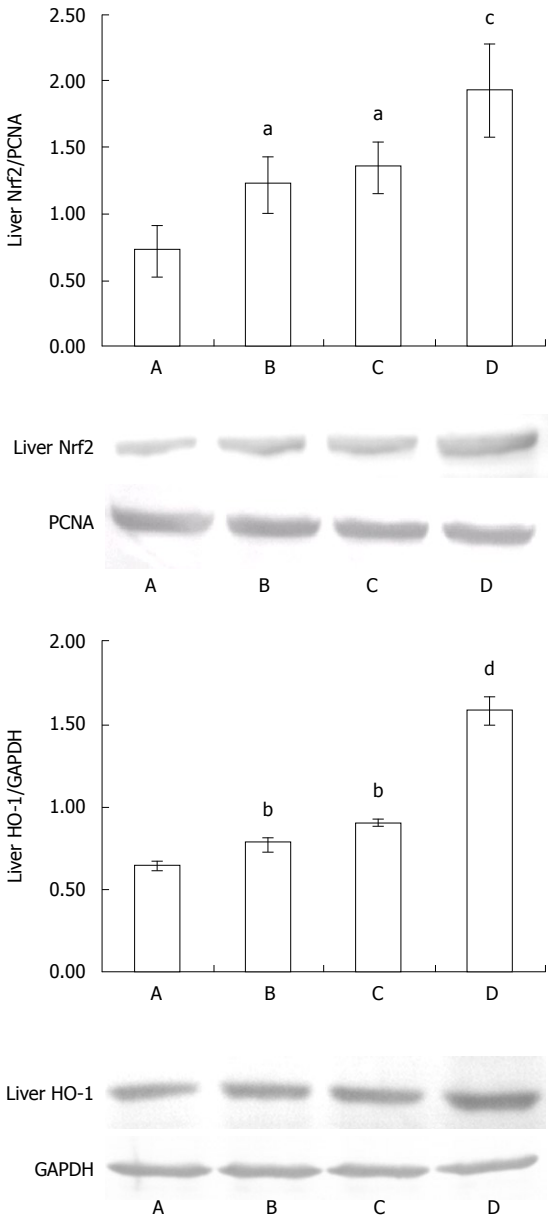


Figure 9 Western blotting analysis of expression of liver Nrf2 and HO-1 in the control (A), SFN control (B), intestinal I/R (C) (1 h ischemia and 2 h reperfusion) and SFN pretreatment (D) groups. PCNA and GAPDH were as the internal control respectively. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* intestinal I/R group.

In conclusion, ROS plays an important role in the pathogenesis of liver injury induced by intestinal I/R. SFN exerted the protective effect on the liver injury induced by intestinal I/R, attributable to the antioxidant effect through Nrf2-ARE pathway.

ACKNOWLEDGMENTS

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COMMENTS

Background

Sulforaphane (SFN) is a natural product derived from isothiocyanate which is present in cruciferous vegetables such as broccoli that has been used as a chemopreventive compound. The cytoprotective effect exerted by this compound is mediated by transcription factor NF-E2-related factor-2 (Nrf2), which binds to the antioxidant response element (ARE) in the promoter region of a number of genes, encoding for antioxidative and phase 2 enzymes. SFN can disrupt the Nrf2-Keap1 complex, and permit Nrf2 to translocate into the nucleus to activate the ARE-driven genes.

Research frontiers

Studies suggest that Nrf2 activation by SFN results in effective protection from cancers and renal ischemia/reperfusion (I/R), but no one has evaluated SFN to determine if it can protect liver injury induced by intestinal I/R.

Innovations and breakthroughs

The study for the fist time showed that SFN exerted the protective effect on the liver injury induced by intestinal I/R, attributable to the antioxidant effect through Nrf2-ARE pathway.

Applications

This study has indicated that SFN pretreatment attenuates liver injury induced by intestinal I/R in rats, attributable to the antioxidant effect through Nrf2-ARE pathway.

Terminology

Nrf2 is sequestered in the cytoplasm by Keap1, and upon exposure of cells to inducers such as oxidative stress and certain chemopreventive agents, Nrf2 dissociates from Keap1, translocates to the nucleus, binds to ARE, and transactivates phase 2 detoxifying and antioxidant genes. The activation of Nrf2/ARE pathway attenuates ischemia/reperfusion injury.

Peer review

The paper is well-written and data are convincing. All data confirm the hypothesis and adequately discussed.

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BRIEF ARTICLE

Management of recto-vaginal fistulas after prosthetic reinforcement treatment for pelvic organ prolapse

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Abstract

AIM: To communicate our findings on successful treatment of recto-vaginal fistulas (RVFs) after prosthetic reinforcement surgery of pelvic organ prolapse (POP).

METHODS: A retrospective single center study between 1998 and 2008 was performed. A total of 80 patients with RVF were identified, of which five patients (6%), with a mean age of 65 years (range: 52-73), had undergone previous surgery for POP with prosthetic reinforcement.

RESULTS: All patients complained about ongoing vaginal infections and febrile episodes. These symptoms were reported after a mean period of 18 mo after POP repair. As a first intervention, three patients underwent ablation of the prosthetic material (PM). As a second intervention, open proctectomy with a primary anastomosis, an omental patch, and a protective ileostomy were performed in two patients. One patient required a terminal colostomy due to complete destruction of the anal sphincters. In two other patients, ablation of the PM and proctectomy was performed as a one-step procedure. The postoperative course in all patients was uneventful, with a mean length of hospitalization of 20 d (range: 15-30). Closure of the ileostomy was achieved in all four patients within four months. After a mean period of 35 mo (range: 4-60) of follow-up, no recurrence was observed with normal continence in four patients.

CONCLUSION: In our experience, the definitive treatment of high RVFs after PM repair for POP necessitates ablation of the PM, proctectomy with a primary colorectal anastomosis, an omental patch interposition, and a temporary ileostomy.

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Key words: Pelvic organ prolapse; Recto-vaginal fistula; Prosthetic treatment

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INTRODUCTION

Pelvic organ prolapse (POP) is a significant health issue in women worldwide. Approximately 250 000 procedures are performed annually in the United States alone[1,2]. The search for a permanent cure for POP has been ongoing for more than a century and remains a challenge today. A number of different approaches have been described for POP repair, including abdominal (open, laparoscopic, and robotic) and vaginal techniques. Recently, the use of prosthetic material (PM) in pelvic floor surgery has become increasingly popular due to the high incidence of recurrence with primary repairs and no surrogate material. Nevertheless, our knowledge about the possible benefit of PM reinforcement for POP repair remains limited. Currently, randomized trials support the superiority of techniques using PM for cystocele repair, whereas the results for colpo- and rectocele repair remain unclear or controversial[3,4].

With the use of PM, new and specific complications occur; these include PM infection, PM erosion or shrinkage, and visceral extrusion. In a systematic review of 2653 patients who underwent surgery for apical vaginal prolapse, Feiner *et al*[5] reported mesh erosion as the single most frequent complication in 4.6% to 10.7%, depending on the different PMs used. In a retrospective study of monofilament mesh placement in the anterior and posterior compartment for POP repair, Dwyer *et al*[6] reported a patient that had developed an recto-vaginal fistula (RVF). Two years later, Hilger *et al*[7] published a case study of RVF formation three months after a posterior intravaginal slingplasty and mesh augmented rectocele repair was performed, which ended in a permanent colostomy after two unsuccessful attempts to correct the fistula. Our knowledge on the incidence of RVF formation after prosthetic material repair for POP is still limited. In addition, uncertainty over the best management of this complication remains. This is in part due to the small number of case reports present in the medical literature. Here, we report our experience with POP management; to the best of our knowledge, this is the largest case series in the literature of RVFs after previous reinforcement surgery for POP.

MATERIALS AND METHODS

This was a retrospective single center study between 1998 and 2008. During that period, a total of 80 patients with RVF were treated at the Department of Visceral- and Oncological Surgery, Hôpital Timone, Marseille, France. Five female patients, with a mean age of 65 years (range: 52-73), were identified that had undergone previous surgery for POP with PM reinforcement. All patients had POP surgery done elsewhere. Details on previous POP repair were as follows: one Prolift® procedure (Ethicon Women's Health and Urology, Somerville, NJ) for urinary stress incontinence, one vaginal wall sling procedure (Bologna technique) and a laparoscopic repair with two multifilamentous polypropylene meshes implanted in both cases for a colpocystocele. Two other patients had open transabdominal implantation of a multifilamentous polypropylene mesh for a colpocysto- and a colporectocele, respectively, with the mesh fixed to the sacrospinous ligaments. Three patients were referred to our department after the PM had been removed transperinealy or transabdominaly, with creation of a temporary colostomy in one case.

At our department, all patients underwent a Gadolinium enhanced magnetic resonance imaging (MRI) of the pelvis using T1 and T2 weighted sequences, such as ano-rectoscopy or endorectal ultrasound (EUS), to evaluate the anal sphincter and a full gynecological work-up. Finally, in all cases, a complete colonoscopy to search for other underlying pathologies that could provoke RVF was routinely performed.

All surgical interventions were performed under general anesthesia and antibiotic cover. The definitive surgical therapy at our department consisted of an open proctectomy, colo-rectal anastomosis, an omental patch, and a protective ileostomy. One patient underwent a Hartmann procedure. A closed suction pelvic drainage was used in all patients.

For postoperative follow-up, all patients in our department underwent an initial clinical control (including Wexner score[8]), ano-rectoscopy, gynecological examination, and an MRI of the pelvis six months after final surgery had been performed.

RESULTS

Patient characteristics, details of surgery and follow-up are summarized in Table 1. The mean time interval between POP repair and the onset of ailments was 18 mo (range: 2-48). All five patients complained about vaginal discharge, dyspareunia, repetitive vaginal infections, and febrile episodes. However, in one patient (No. 3) the leading clinical symptom was complete stool incontinence. Patients No. 1, 2, and 3 underwent ablation of the PM before they had been referred to our department. Patient No. 1 had undergone open transabdominal ablation of the PM with creation of a temporary colostomy. Ablation of the PM in patients No. 2 and 3 was done *via* a transperineal or laparoscopic approach, respectively.

Pelvic MRI at our department provided evidence of a high RVF in all five patients. In patient No. 3 a complex low RVF with a destruction of the internal and external anal sphincter complex was additionally diagnosed (Figure 1).

EUS examination was normal in three patients; however, in patients No. 1 and 3, the anal sphincters were found with an inflammatory inhibition or destruction at a circumference of 20° and 180°, respectively. Nevertheless, in all patients, except for one (No. 3, Wexner score 19[8]), the preoperative Wexner score was normal. Diagnostic colonoscopy (including routine biopsies) was normal except for patient No. 5, where the mesh had completely eroded and migrated into the rectum (Figure 2).

The intra- and postoperative course was uneventful in all patients. Ileostomy closure was achieved in all four patients within four months after final surgery. The

Table 1 Patient characteristics and details of surgery and follow-up

Patients No.	Age (yr)	Primary pathology POP surgery/type of PM	Onset of symptoms (mo)	Primary surgery	Delay to secondary surgery (mo)	Secondary surgery	Follow-up (mo)/ Wexner score
1	73	Colpo-cystocele laparoscopic approach/two polypropylene meshes	24	Temporary colostomy + ablation of PM	6	Open proctectomy, omental patch + PI	36/3
2	65	Colpo-cystocele Bologna procedure/ two polypropylene meshes	12	Laparoscopic ablation of PM	12	Open proctectomy, omental patch + PI	36/2
3	60	SUI Prolift® procedure/two polypropylene meshes	6	Transperineal ablation of PM	6	Hartmann	36/NA
4	52	Colpo-rectocele transabdominal approach/one polypropylene mesh	2	-	-	Open proctectomy, omental patch + PI	60/0
5	73	Cysto-colpocele transabdominal approach/one polypropylene mesh	48	-	-	Open proctectomy, omental patch + PI	6/0

POP: Pelvic organ prolapse; NA: Not applicable; PM: Prosthetic material; SUI: Stress urinary incontinence; PI: Protective ileostomy.

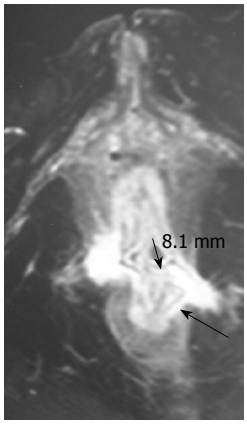


Figure 1 Pelvic magnetic resonance imaging (MRI) showing a transverse section of patient No. 3 with a complex recto-vaginal fistula (RVF) and destruction of the anal sphincter complex. The black arrows show a collection of 8.1 mm with complete destruction of the anal sphincter.

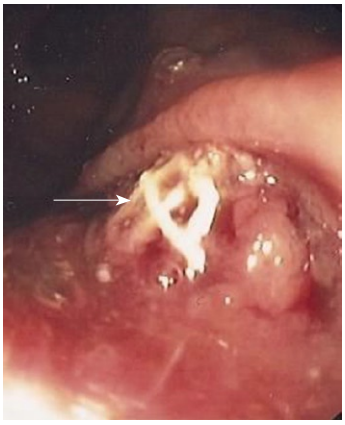


Figure 2 Colonoscopy. Recto-vaginal fistula (arrow) due to polypropylene mesh erosion and migration into the rectum.

mean hospitalization (including closure of the ileostomy) was 20 d (range: 15-40).

After a mean postoperative follow-up period of 34 mo (range: 6-60), no fistula recurrences were observed. In all patients where colo-rectal continuity could have been preserved, only patients No. 1 and 2 suffered from mild postoperative stool incontinence, with Wexner scores of 3 and 2, respectively. Stool continence in patients No. 4 and 5 was found to be completely normal (Wexner 0).

DISCUSSION

POP is a significant health problem in women worldwide, with an estimated 250 000 annual procedures performed in the United States alone[1,2]. These figures are predicted to rise by 45% over the next 30 years due to the increased life expectancy of women in the Western world, and an increasing prevalence of pelvic floor dysfunction with age[9].

Hundreds of techniques have been reported in POP surgery, whether vaginal, abdominal, or laparoscopic, but there is little or no real consensus on which procedure is most appropriate. This is in part due to the lack of high-quality, randomized controlled trials assessing the long-term anatomical and functional outcomes using validated tools and procedures[10].

As traditional surgical techniques for POP utilizing the patient's own tissue do not restore normal anatomy and have a very high failure rate, the use of PM has recently become more frequent. As far as the treatment for cystocele is concerned, better results are reported compared to the traditional surgical approach without use of PM[3,4].

However, with the more liberal use of different types of PM, new adverse effects and complications in patients who underwent POP surgery have been observed. In a public health notification published in October 2008 by the U.S Food and Drug Administration (FDA) it is stated, that over a period of three years, nine different mesh manufacturers reported about 1000 complications associated with transvaginal placement of surgical mesh in repair of POP and SUI. Furthermore, it stated that specific characteristics of patients at increased risk for complications have not been determined. However, contributing factors might include the overall health of the patient, the mesh material, the size and shape of the mesh, the surgical technique used, concomitant procedures undertaken (e.g. hysterectomy), and possibly estrogen status[11].

In our current series of five patients with high RVF after mesh repair of POP, all patients were in good overall health at the time of POP surgery. None of them had previous POP surgery or were under a concomitant medication or medical treatment that could have promoted RVF formation.

The type of mesh implanted has been found to be critical (reported to be pivotal). Mesh erosion through the vaginal epithelium is the most frequently observed mesh-related complication. However, this complication

differs between the different types of meshes used. A high rate of mesh erosion has been reported for non-resorbable synthetic meshes such as Gore-Tex®, Marlex®, and Mersilene®[12].

In our case series, RVF formation was related to multifilamentous polypropylene mesh implantation in all five cases, although the use of polypropylene mesh is currently promoted due to good biocompatability characteristics with a somewhat lower incidence of mesh related complications compared to other mesh types reported in the literature[12,13].

Information concerning RVF formation after mesh surgery for POP is rare in the medical literature. In a retrospective study of monofilament mesh placement in the anterior and posterior compartment for POP repair in 97 patients, Dwyer *et al*[6] observed one patient (1%) who had developed an RVF. In a case report, Hilger *et al*[7] reported an RVF that developed three months after a posterior intravaginal slingplasty and mesh augmented rectocele repair was performed. In another retrospective multicenter study of 687 patients who underwent a vaginal cure with the Prolift® procedure, one patient with an RVF (0.15%) in the postoperative follow-up period was found. However, in the same series, an intraoperative rectal erosion (0.15%), a rectal lesion (0.15%), and a recto-vesical fistula (0.15%) were also reported[14]. The exact incidence of this serious complication remains unknown, and is currently between 0.15% and 1%, as reported in the literature. However, the true incidence of RVF is probably somewhat higher, as the formation of RVFs is very likely the sequel of mesh infection and/or mesh extrusion, which are quite common.

Mesh extrusion after POP is generally observed between four and sixteen months postoperatively[15]. In our study, patients started to complain of vaginal discharge, repetitive vaginal infections, and febrile episodes after a mean period of 18 mo (range: 2-48) after POP repair. Therefore, the symptoms outlined above, even several years after POP surgery, should prompt clinicians to search for an RVF. In all five of our patients, MRI of the pelvis revealed a high RVF and furthermore helped to estimate the extent of pelvic inflammation and tissue destruction. Three patients underwent ablation of the PM before they were referred to our department. In one patient, a temporary colostomy had also been performed at the time of PM ablation. In cases of mesh infection, ablation of the mesh by a minimal invasive procedure, if possible, is currently recommended as a first treatment option[16]. This manoeuvre is generally facilitated by a bacterial biofilm, which isolates the PM from the surrounding tissue[13,17]. The definitive surgical treatment should then be performed after a delay of three to six months after pelvic inflammation has minimized[15]. In cases of a simple RVF, it is thought that once the infected PM has been removed, a spontaneous cicatrisation of the initially inflamed tissue will lead to spontaneous RVF closure[18].

However, in the three patients who underwent ablation of the PM in a primary surgical intervention outside our department, pelvic inflammation did not substantially minimize within 6 to 12 mo. Even protective colostomy did not resolve pelvic inflammation and closure of the RVF. This might be explained, in some part, by the finding that in all three patients some parts of PM were still found in the pelvis during final surgery, which might have promoted the ongoing pelvic inflammatory process. Therefore, it is unlikely that simple ablation of the PM, with or without the creation of a temporary colostomy, will completely resolve pelvic inflammation and lead to a permanent cure. In those two patients who were directly transferred to our department, ablation of the PM, proctectomy, colo-rectal anastomosis, an omental patch, and temporary ileostomy was performed as a one-step procedure. In cases of severe sphincter destruction, a definitive colostomy is the procedure of choice.

RVF after mesh surgery for POP is a rare, but severe, complication. The exact incidence of this complication depends on the type of PM implanted and the type of previous surgeries performed, and is currently about 0.15% to 1%, as reported in the medical literature. However, it is likely that the true incidence is somewhat higher. Ablation of the PM in a first surgical procedure is unlikely to remove all PM and there is a risk that parts of the infected mesh are left in place, which probably promotes an ongoing inflammation of the pelvis. If the general condition of the patient allows, we recommend a radical one-step surgical procedure with open PM ablation, proctectomy, an omental patch, colo-rectal anastomosis, and creation of a temporary ileostomy.

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COMMENTS

Background

An estimated 11% of women will require surgery for pelvic floor dysfunction, with 29% requiring at least a second surgery. To improve upon the results obtained with the use of native tissue, many surgeons have turned to different graft materials, especially synthetic mesh. Polypropylene mesh has been widely used and studied in such procedures, as abdominal sacral colpopexies and numerous mid-urethral slings. Over the last several years, the use of polypropylene mesh has been extended to augment vaginal repair of the anterior and/or posterior vaginal wall. With the use of prosthetic material (PM), new and specific complications have occurred. These include PM infection, PM erosion or shrinkage, and visceral extrusion. In a systematic review of 2 653 patients that underwent surgery for apical vaginal prolapse, Feiner *et al* reported mesh erosion as the single most frequent complication in 4.6% to 10.7%, depending on the different PMs used.

Research frontiers

Knowledge on the incidence of rectovaginal fistula (RVF) formation after prosthetic material repair for pelvic organ prolapse (POP) is still limited. In addition, uncertainly on the best management of this complication remains. This is in part due to the small number of case reports present in the medical literature. Here, the authors report on their experience with POP management, and to the best of their knowledge this is the largest case series the literature of RVFs after previous reinforcement surgery for POP.

Innovations and breakthroughs

In case of a simple RVF, it is thought that once the infected PM has been removed, a spontaneous cicatrisation of the initially inflamed tissue will lead to

spontaneous RVF closure. However, in three patients who underwent ablation of the PM in a primary surgical intervention outside our department, pelvic inflammation did not substantially minimize within 6 to 12 mo. Even protective colostomy did not resolve pelvic inflammation and closure of the RVF. This might be explained, in some part, by the finding that, in all three patients, some parts of the PM were still found in the pelvis during final surgery, which might have promoted the ongoing pelvic inflammatory process. Therefore, it is unlikely that simple ablation of the PM, with or without the creation of a temporary colostomy, will completely resolve pelvic inflammation and lead to a permanent cure.

Applications

RVF after mesh surgery for POP is a rare, but severe and complication. The exact incidence of this complication depends on the type of PM implanted and the type of previous surgeries performed, and is currently about 0.15% to 1%, as reported in the medical literature. However, it is likely that the true incidence is somewhat higher. Ablation of the PM in a first surgical procedure is unlikely to remove all PM and there is a risk that parts of the infected mesh are left in place, which probably promotes an ongoing inflammation of the pelvis. If the general condition of the patient allows, the authors recommend a radical one-step surgical procedure with open PM ablation, proctectomy, an omental patch, colo-rectal anastomosis, and creation of a temporary ileostomy.

Peer review

This is a good report of uncommon problem with clear-cut guidelines for this problem.

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BRIEF ARTICLE

Vitamin D receptor gene polymorphisms and hepatocellular carcinoma in alcoholic cirrhosis

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Abstract

AIM: To assess the relationship between vitamin D receptor (*VDR*) gene polymorphisms and the presence of hepatocellular carcinoma (HCC).

METHODS: Two-hundred forty patients who underwent liver transplantation were studied. The etiologies of liver disease were hepatitis C (100 patients), hepatitis B (37) and alcoholic liver disease (103). A group of 236 healthy subjects served as controls. HCC in the explanted liver was detected in 80 patients. The following single nucleotide gene polymorphisms of the VDR were investigated by polymerase chain reaction and restriction fragment length polymorphism: FokI C>T (*F*/*f*), BsmI A>G (*B*/*b*), ApaI T>G (*A*/*a*) and TaqI T>C (*T*/*t*) (BAT).

RESULTS: The frequencies of genotypes in patients without and with HCC were for FokI *F*/*F* = 69, *F*/*f* = 73, *f*/*f* = 18 and *F*/*F* = 36, *F*/*f* = 36, *f*/*f* = 8; BsmI *b*/*b* = 45, *B*/*b* = 87, *B*/*B* = 28 and *b*/*b* = 33, *B*/*b* = 35, *B*/*B* = 12; for ApaI *A*/*A* = 53, *A*/*a* = 85, *a*/*a* = 22 and *A*/*A* = 27, *A*/*a* = 38, a/a = 15; for TaqI *T*/*T* = 44, *T*/*t* = 88, *t*/*t* = 28 and *T*/*T* = 32, *T*/*t* = 38, *t*/*t* = 10. Carriage of the *b*/*b* genotype of BsmI and the *T*/*T* genotype of TaqI was significantly associated with HCC (45/160 *vs* 33/80, $P < 0.05$ and 44/160 *vs* 32/80, $P < 0.05$, respectively). The absence of the A-T-C protective allele of BAT was significantly associated with the presence of HCC (46/80 *vs* 68/160, $P < 0.05$). A strong association was observed between carriage of the *BAT* A-T-C and G-T-T haplotypes and HCC only in alcoholic liver disease (7/46 *vs* 12/36 *vs* 11/21, $P < 0.002$, respectively).

CONCLUSION: *VDR* genetic polymorphisms are significantly associated with the occurrence of HCC in patients with liver cirrhosis. This relationship is more specific for patients with an alcoholic etiology.

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Key words: Alcohol; Hepatocellular carcinoma; Liver cirrhosis; Vitamin D receptor polymorphisms

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INTRODUCTION

Hepatocellular carcinoma (HCC), the fifth most common cause of cancer and the third leading cause of cancer-related death worldwide[1], accounts for 85% to 90% of primary liver cancers. It is characterized by dynamic temporal trends and marked demographic, geographic and ethnic variations[1]. In western countries, HCC is most often superimposed on cirrhosis, its major risk factor[2,3]. Alcohol, hepatitis B virus (HBV) and hepatitis C virus (HCV) are the main etiologic agents of cirrhosis and HCC worldwide. HBV and HCV supposedly exert a direct carcinogenic effect, accounting for the high prevalence of liver cancer among infected patients[2,3]; the same might not be true for alcohol[1]. Overall, it is prudent to affirm that differences in the incidence rates and the strong gender distribution in HCC are not entirely due to differences in the exposure to the three causative agents mentioned above. Genetic factors could also contribute, particularly gene polymorphisms of inflammatory cytokines and growth factor ligands and receptors[4].

The vitamin D receptor (VDR) is a member of the nuclear receptor super-family of ligand-inducible transcription factors which are involved in many physiological processes, including cell growth and differentiation, embryonic development and metabolic homeostasis. The transcriptional activity of this receptor is modulated by ligands, such as steroids, retinoids and other lipid-soluble compounds, and by nuclear proteins acting as co-activators and co-repressors[5,6]. The liganded VDR heterodimerizes with the retinoid X receptor and binds to vitamin D response elements in the promoter of target genes, thereby affecting their transcription. The genomic organization of the *VDR* at locus 12q13.1 shows that the *VDR* gene itself is quite large (over 100 kb) and has an extensive promoter region capable of generating multiple tissue-specific transcripts[7].

Several single nucleotide restriction fragment length polymorphisms have been described in the *VDR* gene in association with neoplastic and non-neoplastic diseases. In particular, *VDR* polymorphisms have been related to, although with conflicting observations, cancers of the breast, prostate, skin, colon-rectum, bladder and kidney[8-10]. Furthermore, *VDR* polymorphisms were found to influence the prognosis of prostate and breast cancer, renal cell carcinoma and malignant melanoma[11]. *VDR* polymorphisms have been investigated in the context of some chronic liver diseases, such as primary biliary cirrhosis and auto-immune hepatitis[12-16]. Surprisingly, however, there are no data in the literature on the possible association between *VDR* polymorphisms and the occurrence of HCC.

The aims of the present paper were (a) to investigate the possible relationship between *VDR* polymorphisms and the occurrence of HCC in patients with liver cirrhosis and (b) to assess whether the etiology of their liver disease exerts a role in modulating such a relationship.

MATERIALS AND METHODS

Patients

The study included 240 consecutive patients who underwent liver transplantation (LT) for end-stage liver disease due to hepatitis B (n = 37, 15.4%), hepatitis C (n = 100, 41.7%) and alcoholic liver disease (n = 103, 42.9%). The main demographic and clinical characteristics are reported in Table 1. Two-hundred thirty-six healthy community blood donors served as controls. They were 164 males (69.5%) and 72 females (30.5%); the median age was 48 years with a range of 18-77 years. The male gender distribution in the patients and controls (74% *vs* 70%) was similar, whereas these two groups differed in age: 54 ± 8 years *vs* 46 ± 13 years (mean ± SD, $P < 0.0001$). Control subjects did not have any clinical and/or laboratory evidence of liver disease or other major pathological conditions, such as diabetes mellitus. All patients and controls were Caucasian. Informed consent to participate in the study was obtained from each subject in accordance with the Declaration of Helsinki and following the guidelines of our ethical committee. All study participants approved the storage of their frozen DNA specimens for research purposes in our laboratory.

Histology

All total hepatectomy specimens were sectioned at intervals of approximately one cm to search for suspicious focal hepatic lesions. Standard histological staining techniques were applied to confirm the presence of HCC and to evaluate the characteristics of the identified tumors, such as pathologic tumor grade (Edmondson grade) and macro- or micro-vascular invasion.

Vitamin D assay

In 113 patients (47.1%), serum samples, which were collected the day before the transplant operation and were separated and stored at -80℃ until analysis, were available to assay the pre-LT serum vitamin D concentration. Circulating 25-hydroxyvitamin D levels were measured using a chemo-luminescent immunoassay implemented on a Liaison automatic analyzer (DiaSorin Inc, Stillwater, MN, USA). Data were expressed in ng/mL. Reference values of serum vitamin D adopted in this study were in accordance with those proposed by the Scientific Advisory Committee of Nutrition[17], which considers serum vitamin D levels < 10-15 ng/mL to be inadequate for bone and overall health in healthy individuals.

Molecular biology

Genomic DNA was isolated from whole blood using the QIAamp DNA Blood Mini Kit (Qiagen, Milan, Italy) according to the manufacturer's instructions. Four diallelic polymorphisms of the *VDR* were genotyped: FokI C>T

Table 1 Main demographic and clinical characteristics of liver transplant recipients (*n* = 240) according to the presence (*n* = 80, 33.3%) or absence (*n* = 160, 66.7%) of HCC

		HCC present (*n* = 80)	HCC absent (*n* = 160)	*P* value
Male gender	178 (74.2)	70	108	< 0.0010
Age[1] (yr)	55 (22-68)	57.2 ± 5.9	52.0 ± 8.6	< 0.0001
Body mass index[1] (kg/m^2)	25.2 (14.8-48.5)	26.4 ± 4.2	24.7 ± 3.3	< 0.0010
Etiology				
Viral (*n* = 137)		50	87	NS
HBV	37 (15.4)			
HCV	100 (41.7)			
Alcoholic	103 (42.9)			
Child-Pugh score[1]	8 (5-14)	7 (5-14)	8 (5-13)	< 0.0001
Child-Pugh score[1] > 8 (*n* = 87)		22	65	< 0.0500
Diabetes mellitus	67 (27.9)	29	38	< 0.0500

The continuous variables are reported as means (standard deviation), whereas the categorical variables are reported as frequencies (%). Statistical analysis was performed using the Student's *t*-test for continuous variables and the Pearson χ^2 test for categorical variables. [1]At transplant operation. HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus; HCV: Hepatitis C virus; NS: Not significant.

(rs10735810) and TaqI T>C (rs10735810) polymorphic sites on the coding sequence, BsmI A>G (rs1544410) and ApaI G>T (rs7975232) on the last intron. For the detection of the *VDR* polymorphisms, the polymerase chain reaction (PCR) technique was applied and followed by restriction fragment length polymorphism assays. The PCR amplifications were carried out in a total volume of 10 μL containing 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 0.01% Tween-20, 0.2 mmol/L deoxyribonucleotides, 2-4 pmol of each primer, 2.0 mmol/L $MgCl_2$ and 0.5 U hot-start *Taq* DNA polymerase (RighTaq, Euroclone, Milan, Italy). The sequences of primers used for FokI were (*f*) 5'-TGCAGCCTTCACAGGTCATA-3', (*r*) 5'-GGCCTGCTTGCTGTTCTTAC-3'; for TaqI and ApaI were (*f*) 5'-ACGTCTGCAGTGTGTTGGAC-3', (*r*) 5'-TCACCGGTCAGCAGTCATAG-3'; for BsmI were (*f*) 5'-CAGTTCACGCAAGAGCAGAG-3', (*r*) 5'-ACCTGAAGGGAGACGTAGCA-3'. All the primers were newly designed with the aid of the NCBI Primer-Blast Tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The cycling conditions for all the *VDR* polymorphisms were set as 40 cycles at 95℃ for 30 s, 61℃ for 30 s and 72℃ for 1 min. In a total volume of 20 μL, amplified DNA (10 μL) was digested overnight with 2 U of restriction endonucleases using the buffers and temperatures recommended by the manufacturers. The presence of restriction sites for the FokI, TaqI, BsmI and ApaI enzymes were coded as '*f*', '*t*', '*b*' and '*a*' and the absence of restriction sites as '*F*', '*T*', '*B*' and '*A*', respectively. The FokI C>T (*F*/*f*) polymorphism was analyzed by digestion of a 157-bp PCR product with FokI (New England Biolabs, Hitchin, UK), which resulted in two fragments of 121 and 36 bp in the presence of the '*f*' allele and in an uncut fragment in the presence of the '*F*' allele. The TaqI T>C (*T*/*t*) and ApaI T>G (*A*/*a*) polymorphisms were analyzed by digestion of a 211-bp PCR product with TaqI (New England Biolabs, Hitchin, UK), which resulted in two fragments of 172 and 39 bp in the presence of the '*t*' allele. These same polymorphisms were also analyzed by digestion with ApaI (New England Biolabs, Hitchin, UK), which resulted in two fragments of 121 and 90 bp for the '*a*' allele. The BsmI A>G (*B*/*b*) polymorphism was analyzed by digestion of a 236-bp PCR product with BsmI (New England Biolabs, Hitchin, UK), which resulted in two fragments of 197 and 39 bp in the presence of the '*b*' allele. All PCR reactions were carried out in a Techne TC-412 thermal cycle, and PCR products were sized by electrophoresis on a 3% agarose gel stained with ethidium bromide.

Statistical analysis

The statistical analysis of data was performed using the BMDP Dynamic Statistical Software Package 7.0 (Statistical Solutions, Cork, Ireland). Continuous variables were presented as median (range) or mean ± SD, whereas categorical variables were expressed as frequencies (%). Differences between continuous variables were assessed by the Student's *t*-test, whereas differences between categorical variables were evaluated using the Pearson χ^2 test. The χ^2 *G* test "Goodness of Fit" was employed to verify whether the proportions of the four polymorphisms were distributed in controls and in patients in accordance with the Hardy-Weinberg equation. Differences in the allelic and genotypic frequencies between different groups were assessed by means of the Pearson χ^2 test and calculation of odds ratios with 95% confidence intervals (CI). Haplotype reconstruction from population genotype data and inferred phased diplotype calculation for each control subject or patient with liver cirrhosis were performed by means of the ARLEQUIN integrated software package for population genetics, version 3.1[18]. Analysis of molecular variance (AMOVA) with a global and a pair-wise approach was performed to assess whether haplotype allelic content differed among groups. Locus-by-locus AMOVA was utilized to assess the statistical contribution of each polymorphism. Pairwise differences in haplotype frequencies were assessed using the exact test for sample differentiation. Linkage disequilibrium between the four analyzed VDR poly-

Table 2 Allelic and genotypic frequencies of the VDR FokI C>T (*F*/*f*), BsmI A>G (*B*/*b*), ApaI T>G (*A*/*a*) and TaqI T>C (*T*/*t*) polymorphisms in controls and liver cirrhosis patients *n* (%)

VDR	Control subjects (*n* = 236)	Liver cirrhosis (*n* = 240)	OR	95% CI	*P*[1]
	F = 0.646	*F* = 0.665	1	Ref.	
FokI	*f* = 0.354	*f* = 0.335	1.085	0.831-1.417	NS
	B = 0.392	*B* = 0.421	1	Ref.	
BsmI	*b* = 0.608	*b* = 0.579	1.127	0.870-1.460	NS
	A = 0.570	*A* = 0.590	1	Ref.	
ApaI	*a* = 0.430	*a* = 0.410	1.084	0.838-1.402	NS
	T = 0.608	*T* = 0.579	1	Ref.	
TaqI	*t* = 0.392	*t* = 0.421	0.887	0.685-1.149	NS
	F/*F* = 104 (44.1)	*F*/*F* = 105 (43.8)	1	Ref.	
FokI	*F*/*f* = 97 (41.1)	*F*/*f* = 109 (45.4)	1.113	0.758-1.635	NS
	f/*f* = 35 (14.8)	*f*/*f* = 26 (10.8)	0.736	0.416-1.303	NS
	B/*B* = 41 (17.4)	*B*/*B* = 40 (16.7)	1	Ref.	
BsmI	*B*/*b* = 103 (43.6)	*B*/*b* = 122 (50.8)	1.214	0.732-2.014	NS
	b/*b* = 92 (39.0)	*b*/*b* = 78 (32.5)	0.869	0.513-1.473	NS
	A/*A* = 76 (32.2)	*A*/*A* = 80 (33.3)	1	Ref.	
ApaI	*A*/*a* = 117 (49.6)	*A*/*a* = 123 (51.3)	0.999	0.668-1.494	NS
	a/*a* = 43 (18.2)	*a*/*a* = 37 (15.4)	0.817	0.477-1.400	NS
	T/*T* = 89 (37.7)	*T*/*T* = 76 (31.7)	1	Ref.	
TaqI	*T*/*t* = 109 (46.2)	*T*/*t* = 126 (52.5)	1.354	0.909-2.017	NS
	t/*t* = 38 (16.1)	*t*/*t* = 38 (15.8)	1.171	0.681-2.013	NS

The odds ratios were constructed with the wild type for each polymorphism as the reference. The statistical analysis was carried out using the Pearson χ^2 test. [1]Pearson χ^2 test; OR: Odds ratio; CI: Confidence interval; VDR: Vitamin D receptor.

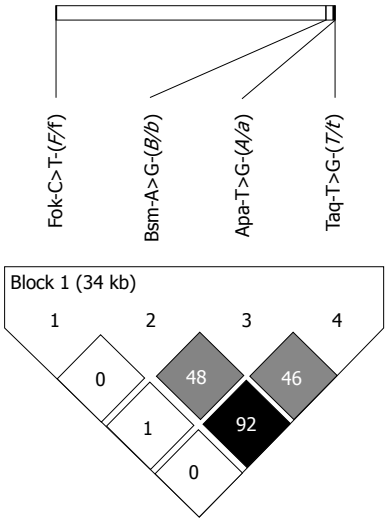


Figure 1 Schematic representation of linkage disequilibrium in the studied population (*n* = 476) between the four VDR polymorphisms: FokI C>T (*F*/*f*), BsmI A>G (*B*/*b*), ApaI T>G (*A*/*a*) and TaqI T>C (*T*/*t*). R^2 for linkage disequilibrium between each marker is reported. Shades of gray are proportional to the R^2 value, expressing the strength of the linkage disequilibrium.

morphisms in the studied population was determined by means of the Haploview software[19]. Stepwise logistic regression analysis with a forward approach was used to verify whether the absence or presence of specific VDR haplotypes was an independent predictor of HCC.

RESULTS

Liver histology

HCC foci were detected in the native livers from 80 (33.3%) of the patients with end-stage liver disease; 33 patients (41.2%) were HCV positive, 17 (21.2%) HBV positive, and 30 (29.1%) had alcoholic liver disease. Table 1 illustrates the distribution of the values of the main demographic and clinical variables of cirrhotic patients according to the presence of HCC. Sixteen patients (20.0%) had an Edmondson grade of one, forty-eight patients (60.0%) had a grade of two, fifteen patients (18.8%) had a grade of three and one patient (1.2%) had a grade of four. Macro- and micro-vascular invasion was only observed in five cases (6.2%).

VDR polymorphisms in liver cirrhosis and controls

FokI C>T (*F*/*f*), BsmI A>G (*B*/*b*), ApaI T>G (*A*/*a*) and TaqI T>C (*T*/*t*) allele and genotype frequencies are reported in Table 2. No departure from the Hardy-Weinberg equilibrium equation was observed for each polymorphism in patients or controls. No significant difference was detected between patients and controls in allele or genotype frequencies. A strong linkage disequilibrium was detected between BsmI and TaqI (R^2 = 0.92); linkage disequilibrium was also detected between ApaI and TaqI (R^2 = 0.46) and between BsmI and ApaI (R^2 = 0.48) (Figure 1).

VDR polymorphisms in patients and controls in relationship to the etiology of liver disease

AMOVA was performed by grouping the liver cirrhosis patients according to the etiology of their liver disease: patients with cirrhosis of viral origin (*n* = 137) and patients with cirrhosis of alcoholic origin (*n* = 103); a third group consisted of control subjects (*n* = 236). A significant difference was detected among these three populations ($P < 0.05$) by global AMOVA; the comparison of

Table 3 Estimated VDR haplotype frequencies [FokI C>T (*F/f*), BsmI A>G (*B/b*), ApaI T>G (*A/a*) and TaqI T>C (*T/t*) polymorphisms] in control subjects and in patients with liver cirrhosis of viral and alcoholic origin

Control subjects (n = 236)			Alcoholic cirrhosis (n = 103)			Viral cirrhosis (n = 137)		
Haplotypes	n	Frequencies	Haplotypes	n	Frequencies	Haplotypes	n	Frequencies
C-A-T-C	139	0.295	C-A-T-C	59	0.286	C-A-T-C	103	0.376
T-G-G-T	102	0.216	T-G-G-T	46	0.223	T-G-G-T	56	0.204
C-G-G-T	99	0.210	C-G-G-T	50	0.243	C-G-G-T	43	0.158
C-G-T-T	58	0.123	C-G-T-T	29	0.141	C-G-T-T	25	0.091
T-A-T-C	41	0.087	T-A-T-C	11	0.053	T-A-T-C	23	0.084
T-G-T-T	23	0.049	T-G-T-T	6	0.029	T-G-T-T	19	0.069
C-A-T-T	4	0.008	C-A-T-T	1	0.005	C-A-T-T	3	0.011
C-G-T-C	3	0.006	C-G-T-C	2	0.010	C-G-T-C	2	0.007
C-G-G-C	2	0.004	C-A-G-C	2	0.010			
T-A-T-T	1	0.002						

The statistical analysis was performed by means of the exact test for sample differentiation based on haplotype frequencies. Alcoholic *vs* viral liver cirrhosis $P < 0.05$.

pairs of population samples demonstrated a significant difference between patients with cirrhosis of viral and alcoholic origin ($P < 0.01$). Locus-by-locus AMOVA showed the following significant differences for the single nucleotide gene polymorphisms tested: FokI C>T (*F/f*) $P = 0.720$, BsmI A>G (*B/b*) $P = 0.047$, ApaI T>G (*A/a*) $P = 0.052$ and TaqI T>C (*T/t*) $P = 0.065$. Estimated VDR haplotype frequencies of FokI C>T, BsmI A>G, ApaI T>G and TaqI T>C polymorphisms are reported in Table 3. A significant difference in haplotype frequencies was detected between patients with cirrhosis of viral and alcoholic origin.

VDR polymorphisms in liver cirrhosis with and without HCC

Table 4 illustrates the genotype frequencies of FokI C>T (*F/f*), BsmI A>G (*B/b*), ApaI T>G (*A/a*) and TaqI T>C (*T/t*) polymorphisms in patients with liver cirrhosis grouped according to the presence (n = 80) or absence (n = 160) of HCC. Patients with HCC were more likely to carry the *b/b* genotype compared with the *B/B* + *B/b* genotypes of the BsmI A>G (*B/b*) polymorphism and the *T/T* genotype compared with the *T/t* + *t/t* genotypes of the TaqI T>C (*T/t*) polymorphism. Considering the BsmI A>G (*B/b*), ApaI T>G (*A/a*) and TaqI T>C (*T/t*) (*BAT*) polymorphisms, carriage of the A-T-C haplotype was associated with the absence of HCC and carriage of the G-T-T haplotype with the presence of HCC. We then grouped the patients as follows: group (a) carriers of the *BAT* A-T-C haplotype (n = 126), group (b) carriers of both *BAT* A-T-C and G-T-T haplotypes or of none of the two haplotypes (n = 75) and group (c) carriers of the *BAT* G-T-T haplotype (n = 39). A significantly linear trend for increasing frequencies of HCC was detected starting with group (a) (34/126, 27.0%) to group (b) (29/75, 38.7%) to group (c) (17/39, 43.6%, $P < 0.05$). Stepwise logistic regression analysis was performed to verify whether carriage of the *BAT* A-T-C and G-T-T haplotypes was a predictor of HCC independent of gender, age (≤/> 50 years), body mass index (</≥ 25 kg/m^2), Child-Pugh score (≤/> 8) at the transplant operation, viral etiology of liver disease and presence of diabetes mellitus. The analysis confirmed that carriage of the *BAT* A-T-C and G-T-T haplotypes was a predictor of HCC occurrence (improvement of χ^2 $P < 0.05$, OR, 1.95, 95% CI: 1.06-3.57) independent of age > 50 years (improvement of χ^2 $P < 0.001$, OR 3.81, 95% CI: 1.75-8.29), male gender (improvement of χ^2 $P = 0.001$, OR 4.01, 95% CI: 1.80-8.96), viral etiology of liver disease (improvement of χ^2 $P < 0.05$, OR 2.19, 95% CI: 1.18-4.05) and body mass index ≥ 25 kg/m^2 (improvement of χ^2 $P < 0.05$, OR 1.82, 95% CI: 0.99-3.35).

VDR polymorphisms in liver cirrhosis with and without HCC in relationship to the etiology of liver disease

AMOVA was performed by grouping the patients with liver cirrhosis according to the etiology of their liver disease (viral origin n = 137 and alcoholic origin n = 103) and the presence (n = 80) or absence (n = 160) of HCC. A significant difference was detected among these four populations ($P < 0.002$); the comparison of pairs of population samples demonstrated significant differences between patients with liver cirrhosis of alcoholic origin with HCC *vs* (a) patients with liver cirrhosis of viral origin with HCC ($P < 0.0001$), (b) patients with liver cirrhosis of viral origin without HCC ($P < 0.0001$) and (c) patients with liver cirrhosis of alcoholic origin without HCC ($P < 0.01$). Locus-by-locus AMOVA showed the following significant differences for the single polymorphisms: FokI C>T (*F/f*) $P = 0.923$, BsmI A>G (*B/b*) $P = 0.000$, ApaI T>G (*A/a*) $P = 0.033$ and TaqI T>C (*T/t*) $P = 0.000$. Estimated VDR haplotype frequencies of FokI C>T, BsmI A>G, ApaI T>G and TaqI T>C polymorphisms are reported in Table 5. A significant difference in haplotype frequencies was detected between patients with alcoholic liver cirrhosis with HCC *vs* patients with viral liver cirrhosis without HCC ($P < 0.01$) and patients with viral liver cirrhosis with HCC ($P < 0.05$). Although no relationship was detected between carriage of the *BAT* A-T-C and G-T-T haplotypes and the pres-

Table 4 Genotypic frequencies of the VDR FokI C>T (*F/f*), BsmI A>G (*B/b*), ApaI T>G (*A/a*) and TaqI T>C (*T/t*) polymorphisms in liver cirrhosis patients grouped according to the presence or absence of HCC

VDR	HCC absent (*n* = 160)	HCC present (*n* = 80)	OR	95% CI	*P*[1]
	F/F = 69 (43.1)	*F/F* = 36 (45.0)	1	Ref.	
FokI	*F/f* = 73 (45.6)	*F/f* = 36 (45.0)	0.945	0.537-1.663	NS
	f/f = 18 (11.3)	*f/f* = 8 (10.0)	0.852	0.345-2.113	NS
	B/B = 28 (17.5)	*B/B* = 12 (15.0)	1	Ref.	[2]
BsmI	*B/b* = 87 (54.4)	*B/b* = 35 (43.8)	0.939	0.433-2.028	NS
	b/b = 45 (28.1)	*b/b* = 33 (41.2)	1.711	0.766-3.813	NS
	A/A = 53 (33.1)	*A/A* = 27 (33.8)	1	Ref.	
ApaI	*A/a* = 85 (53.1)	*A/a* = 38 (47.5)	0.878	0.483-1.595	NS
	a/a = 22 (13.8)	*a/a* = 15 (18.7)	1.338	0.605-2.968	NS
	T/T = 44 (27.5)	*T/T* = 32 (40.0)	1	Ref.	[3]
TaqI	*T/t* = 88 (55.0)	*T/t* = 38 (47.5)	0.594	0.329-1.072	0.08
	t/t = 28 (17.5)	*t/t* = 10 (12.5)	0.491	0.212-1.141	0.09

The odds ratios were constructed with the wild type for each polymorphism as the reference. The statistical analysis was carried out using the Pearson χ^2 test. [1]Pearson χ^2 test; [2]*B/B* + *B/b* genotypes *vs* *b/b* genotype: $P < 0.05$; [3]*T/t* + *t/t* genotypes *vs* *T/T* genotype: $P < 0.05$.

Table 5 Estimated VDR haplotype frequencies [FokI C>T (*F/f*), BsmI A>G (*B/b*), ApaI T>G (*A/a*) and TaqI T>C (*T/t*) polymorphisms] in patients with liver cirrhosis of viral and alcoholic origin according to the presence of HCC

Alcoholic cirrhosis (*n* = 103)						Viral cirrhosis (*n* = 137)					
HCC present (*n* = 30)			HCC absent (*n* = 73)			HCC present (*n* = 50)			HCC absent (*n* = 87)		
Haplotypes	*n*	Frequencies	Haplotypes	*n*	Frequencies	Haplotypes	*n*	Frequencies	Haplotypes	*n*	Frequencies
C-A-T-C	9	0.150	C-A-T-C	50	0.343	C-A-T-C	40	0.40	C-A-T-C	64	0.368
T-G-G-T	16	0.267	T-G-G-T	30	0.205	T-G-G-T	15	0.15	T-G-G-T	38	0.218
C-G-G-T	18	0.300	C-G-G-T	32	0.219	C-G-G-T	19	0.19	C-G-G-T	27	0.155
C-G-T-T	14	0.233	C-G-T-T	15	0.103	C-G-T-T	5	0.05	C-G-T-T	17	0.098
T-A-T-C	1	0.017	T-A-T-C	10	0.068	T-A-T-C	7	0.07	T-A-T-C	15	0.086
T-G-T-T	2	0.033	T-G-T-T	4	0.027	T-G-T-T	11	0.11	T-G-T-T	11	0.063
			C-A-T-T	1	0.007	C-A-T-T	2	0.02	C-G-T-C	1	0.006
			C-G-T-C	2	0.014	C-G-T-C	1	0.01	T-A-T-T	1	0.006
			C-G-G-C	2	0.014						

The statistical analysis was performed by means of the exact test for sample differentiation based on haplotype frequencies. Non-viral liver cirrhosis with HCC *vs* (a) viral liver cirrhosis without HCC, $P < 0.01$, (b) viral liver cirrhosis with HCC, $P < 0.05$.

ence of HCC in patients with viral liver disease, a strong association was observed between carriage of the *BAT* A-T-C and G-T-T haplotypes and HCC in alcoholic liver disease (27/80 *vs* 17/39 *vs* 6/18, P = NS) (7/46 *vs* 12/36 *vs* 11/21, $P < 0.002$).

Vitamin D levels and VDR haplotypes in relationship to HCC occurrence

A significant linear trend was detected when patients were grouped according to gender and vitamin D serum levels (cut-off level 15 ng/mL) in relation to HCC occurrence. Group A and B comprised female patients with vitamin D serum levels ≤ 15 ng/mL and > 15 ng/mL, respectively; group C and D comprised male patients with serum levels of vitamin D ≤ 15 ng/mL and > 15 ng/mL, respectively. HCC was detected with increasing frequency starting with group A (0/12, 0.0%) to group B (2/12, 16.7%) to group C (16/38, 42.1%) to group D (26/51, 51.0%, $P < 0.0005$). A synergistic effect was found between vitamin D serum levels > 15 ng/mL and carriage of the *BAT* ATC haplotype; in these patients, HCC occurred in 5/26 (19.2%) cases compared with 39/87 (44.8%) of the remaining cases ($P < 0.02$).

DISCUSSION

In the present study, patients with liver cirrhosis were found to present allele and genotype frequencies of FokI C>T (*F/f*), BsmI A>G (*B/b*), ApaI T>G (*A/a*) and TaqI T>C (*T/t*) polymorphisms close to those observed in control subjects. Associations between specific polymorphisms and auto-immune hepatitis and primary biliary cirrhosis have been observed in several studies, although with conflicting results. In fact, the presence of the *B* allele of the BsmI A>G (*B/b*) polymorphism was found to be associated with the occurrence of primary biliary cirrhosis in three studies involving Caucasian and Japanese populations[12-14], whereas in two other studies performed in Caucasian and Chinese populations[15,16], primary biliary cirrhosis was related to the presence of the *b* allele. Similarly, Vogel *et al*[15] found an association between chronic auto-immune hepatitis and the *F* allele of the FokI C>T

(*F*/*f*) polymorphism, whereas the opposite results were observed by Fan *et al*[16]. Our study of patients affected by liver cirrhosis of viral (HBV or HCV) and alcoholic origin did not demonstrate any differences in the allele and genotype frequencies of the *VDR* polymorphisms between the patients and controls. The data agree with the only report in the literature on *VDR* polymorphisms in patients with chronic liver disease of a non-auto-immune origin. In fact, Suneetha *et al*[20] did not demonstrate differences in *VDR* polymorphism allele frequencies between controls and patients with chronic viral hepatitis due to HBV.

Some novel findings were provided by the present investigation. The first is represented by the observation of differences in the *VDR* polymorphisms in patients with liver cirrhosis in relationship to the etiology of their liver disease. The analysis of molecular variance found a significant difference in allele frequencies between patients with viral (HBV and HCV) and alcoholic liver cirrhosis; this was accounted for by the significant differences of the BsmI A>G (*B*/*b*), ApaI T>G (*A*/*a*) and TaqI T>C (*T*/*t*) (*BAT*) polymorphisms for which a strong linkage disequilibrium was detected. On the contrary, the FokI C>T (*F*/*f*) polymorphism was non-significantly distributed between these two groups. Consequently, haplotype analysis highlighted a different distribution between the two groups with liver cirrhosis; the *BAT* A-T-C haplotype was more represented in patients with viral cirrhosis (0.460%) than in those with alcoholic cirrhosis (0.339%). We are unable to provide a clear explanation of this previously unreported finding especially because the BsmI A>G (*B*/*b*), ApaI T>G (*A*/*a*) and TaqI T>C (*T*/*t*) (*BAT*) polymorphisms are located in a *VDR* gene region of unknown function. Two possible explanations are hypothesized: (a) the real genetic risk for liver cirrhosis might be in linkage disequilibrium with the observed haplotypes of these polymorphisms and/or (b) gene-environment interactions have a causative role.

The second novel finding of this paper is the significant association between *VDR* polymorphisms and the presence of HCC in patients with liver cirrhosis. HCC was found to be associated with the *b* allele of the BsmI A>G (*B*/*b*) polymorphism and with the *T* allele of the TaqI T>C (*T*/*t*) polymorphism. The *BAT* A-T-C haplotype was inversely related to the occurrence of HCC, whereas the *BAT* G-T-T haplotype was directly associated with this cancer; these associations were independent of the main demographic and clinical variables known to be strong predictors of the occurrence of HCC. *VDR* polymorphisms have been explored in cancers of epithelial origin, such as breast, ovarian, prostate, lung and skin cancers[20,21]. Even though some studies did not detect associations between the *VDR* polymorphisms and these diseases, e.g. Gsur *et al*[23] in prostate cancer and Dunning *et al*[24] in breast cancer, the majority of the authors found a significant association between the *VDR* polymorphisms and cancer[8-10,20]. In particular, carriage of the *B* BsmI A>G (*B*/*b*) allele and of the *t* TaqI T>C (*T*/*t*) allele has been described to exert a protective effect in prostate cancer[24], malignant melanoma[25,26] and breast cancer[27]; these results support our findings in the present series on HCC in liver cirrhosis.

The third observation that may be derived from these data concerns the strong interaction between the presence of HCC and the etiology of liver cirrhosis in relationship to *VDR* polymorphisms. Both the analysis of molecular variance and the estimated haplotype frequencies highlighted the different behaviors that were detected in the *VDR* polymorphisms in alcoholic patients with HCC, patients with alcoholic cirrhosis without HCC and patients with liver cirrhosis of viral origin complicated or uncomplicated by the presence of HCC. Locus-by-locus analysis demonstrated that the major contribution was provided by the BsmI A>G (*B*/*b*) and TaqI T>C (*T*/*t*) polymorphisms; the *BAT* A-T-C haplotype was strongly protective, whereas the G-T-T haplotype was associated with HCC in patients with alcoholic cirrhosis but not in patients with liver cirrhosis of viral origin.

Besides the classic action involving calcium and phosphate homeostasis, vitamin D possesses non-classic actions also known to be mediated through the VDR[28]. First, vitamin D exerts immune modulation activity either by stimulating innate immune function[29] and/or by inhibiting hyper-activity of adaptive immunity[30]. More closely related to the subject of the present study, vitamin D has been extensively evaluated for its potential anticancer activity in animal and cell studies. It has been suggested that the anticancer activity of vitamin D is due to its anti-proliferative and pro-differentiating action in most cell types[31]. From an epidemiological point of view, there is some evidence that low vitamin levels are associated with a higher incidence of cancer[32] even though there is no known beneficial effect of treating cancers with vitamin D[33].

Patients with liver cirrhosis are known to be at high risk for vitamin D deficiency in direct proportion to the severity of their chronic liver disease[34,35]. This observation applies particularly to cirrhotic patients with end-stage liver cirrhosis who are subjected to liver transplantation[36], such as those investigated in the present study. In contrast, chronic alcohol abuse is a factor that can interfere in multiple ways with vitamin D metabolism either through malnutrition with an assumption of reduced nutrient intake and/or reduced exposure to sunlight. Reduced vitamin D levels have been detected in the majority of alcoholic patients without chronic liver disease[37]. It is conceivable, therefore, that in patients with liver cirrhosis, the synergistic action of severely reduced serum vitamin D levels and of a specific *VDR* haplotype facilitates HCC development. In fact, supporting this hypothesis, we found vitamin D insufficiency ($\leqslant$ 15 ng/mL) in a large proportion (55.8%) of our patients with end-stage chronic liver disease; moreover, a gender-adjusted association between vitamin D insufficiency and the occurrence of HCC was also detected. Finally, HCC foci were observed less frequently in the native livers of patients carrying the protective *BAT* A-T-C haplotype and simultaneously possessing serum vitamin D levels > 15 ng/mL.

In conclusion, *VDR* genetic polymorphisms are signif-

icantly associated with the occurrence of HCC in patients with liver cirrhosis. This relationship is more specific for patients with an alcoholic etiology.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third leading cause of cancer-related death worldwide. Liver cirrhosis due to alcohol consumption or to chronic infection by hepatitis C virus (HCV) and hepatitis B virus (HBV) are considered the main etiologic agents for the development of HCC. The differences in the incidence rates and the strong gender distribution of HCC are probably not entirely due to differences in exposure to the three causative agents mentioned above. Recently, several genetic factors regarding gene polymorphisms of inflammatory cytokines and growth factors have been considered.

Research frontiers

The vitamin D receptor (VDR) is a member of the nuclear receptor superfamily of ligand- inducible transcription factors which are involved in cell growth and differentiation. Several single nucleotide restriction length polymorphisms have been described in the *VDR* gene in association with cancers of the breast, prostate, colon, bladder and kidney. There are no data in the literature concerning the prevalence of *VDR* polymorphisms in HCC of different etiologies.

Innovations and breakthroughs

This is the first study to examine the potential role of *VDR* genetic polymorphisms in the occurrence of HCC in humans. In this study, the authors investigate the *VDR* single nucleotide gene polymorphisms FokI C>T (*F/f*), BsmI A>G (*B/b*), ApaI T>G (*A/a*) and TaqI T>C (*T/t*) (BAT) in a large series of patients who underwent liver transplantation due to liver cirrhosis with or without HCC. They demonstrate that carriage of the *b/b* genotype of BsmI and the *T/T* genotype of TaqI was significantly associated with HCC, whereas carriage of the *BAT* A-T-C and G-T-T haplotypes was significantly more prevalent in patients with alcoholic liver disease and HCC.

Applications

The characterization of *VDR* genetic polymorphisms in patients with liver cirrhosis could help to identify those who are at high risk of developing HCC. This observation could be used to modify the strategy of periodical ultrasound surveillance in this category of patients.

Peer review

In this paper, the relationships between *VDR* gene polymorphisms and the presence of HCC have been evaluated in patients with viral or alcoholic cirrhosis. The most important result of this study is the observation that some *VDR* genetic polymorphisms are associated with the occurrence of HCC in patients with liver cirrhosis. The results are convincing, well presented and of some interest.

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BRIEF ARTICLE

Glycated hemoglobin and antidiabetic strategies as risk factors for hepatocellular carcinoma

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Abstract

AIM: To evaluate the relationship between glycemic control [assessed by glycated hemoglobin (HbA1c)], antidiabetic therapies and the risk of hepatocellular carcinoma (HCC).

METHODS: We recruited 465 patients with HCC, 618 cases with liver cirrhosis and 490 controls with no liver disease. Among subjects with type 2 diabetes mellitus (DM2), the associations between the antidiabetic strategies and HbA1c level with HCC were determined through 2 series of multivariate logistic regression models using cirrhotic patients and controls as comparison groups.

RESULTS: DM2 prevalence was 31.2% in patients with HCC, 23.2% in cirrhotic patients and 12.6% in controls ($P < 0.0001$). In 86% of study subjects, DM2 had been diagnosed for more than 1 year before the HCC diagnosis. HCC patients with DM2 had a 1.5-2.5-fold increased risk of liver cancer. The HbA1c mean levels were significantly higher in DM2 patients with HCC than in cirrhotic and control DM2 patients. Antidiabetic treatment with metformin was more common among cirrhotic and control DM2 subjects than among cases with HCC. In both series of multivariate analyses, treatment with metformin significantly reduced the risk of HCC by more than 80% compared with sulphonylureas and insulin therapy. No significant differences were seen between sulphonylureas and insulin treatment. Elevated HbA1c levels were positively related to the risk for HCC in diabetic patients, with a 26%-50% increase in risk for each 1% increase in HbA1c values.

CONCLUSION: In patients with preexisting DM2, the risk of HCC is positively associated with poor chronic glycemic control and significantly decreased by metformin therapy.

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Key words: Hepatocellular carcinoma; Type 2 diabetes mellitus; Glycemic control; Metformin therapy; HbA1c level

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INTRODUCTION

The incidence and mortality rates of hepatocellular carci-

noma (HCC) have significantly increased in recent years, particularly in Western countries[1,2]. The main known risk factors for HCC are hepatitis C and B virus (HCV, HBV) infections and chronic alcohol abuse, but at least 25% of HCC cases do not have any recognized etiology. Diabetes mellitus has been proposed to be associated with HCC development[3], and several investigations in recent years clearly indicated that type 2 diabetes mellitus (DM2) is a risk factor for HCC[1,4-9]. During the past 2 decades, the prevalence of diabetes mellitus, and in particular of DM2, has dramatically increased in many countries, including Italy[10]. Sedentary lifestyles, excessive food consumption and obesity appear to be the main causes of the current diabetes mellitus epidemic[11].

It is unclear how DM2 influences hepatocarcinogenesis. Insulin resistance (IR) is a basic feature of DM2, a disorder characterized by hyperglycemia associated with IR and consequent hyperinsulinemia. Several reports have suggested that the mechanism underlying the effects of DM2 and antidiabetic therapies on hepatic carcinogenesis could be related to IR and hyperinsulinemia[12-16]. In DM2 patients, insulin plasma levels are chronically increased by therapies based on both exogenous insulin or insulin secretagogues, such as sulphonylureas. An excess of insulin plasma levels is the proposed mechanism underlying the well established association of DM2[16-18] and obesity[19] (the so called diabesity) with several types of solid tumors[20,21]. In a previous study, we found that DM2 in our population was an independent risk factor for HCC, and that it precedes HCC development[12]. In addition, in male chronic liver disease (CLD) patients with DM2, the risk of HCC is increased by insulin or sulphonylurea treatment. We also reported that the association between IR and CLD began in the early stages of liver fibrosis, and that DM2 significantly increased IR in HCC patients. Therefore, IR together with the consequent hyperinsulinemia, seems to play a major role in the link between DM2 and hepatocarcinoma[13].

Recent reports have shown that liver carcinogenesis is increased not only in HCV infected patients with previously diagnosed and/or treated DM2[22,23], but also in HCV infected subjects with no pre-existing history of DM2 and in the early stages of glucose intolerance, as diagnosed by a 75 g oral glucose tolerance test[24]. In addition, , a positive association between high dietary glycemic load and risk of HCC in patients with chronic HBV and HCV infection has been reported[25]; the pathogenesis and outcome of cryptogenic HCC seems to be more closely associated with the risk factors for metabolic syndrome than with HBV and HCV[26].

Despite extensive literature linking glucose intolerance with hepatocarcinogenesis, it remains unclear whether blood glucose control may influence the malignant potential that DM2 exerts on the liver. Only one prospective study reported that hyperglycemia was associated with increased total cancer risk in women and men, independently of obesity[27]. As far as we know, the effect of glycemic control on liver carcinogenesis and the role of treatment strategies to achieve the metabolic control in DM2 patients with CLD have not been investigated yet.

Therefore, the aims of our study were to explore the association between HCC and DM2 in CLD subjects, and to assess the relationships between glycemic control, as determined by glycated hemoglobin (HbA1c) measurement, antidiabetic strategies and the risk of HCC in DM2 patients with CLD.

MATERIALS AND METHODS

Ethics

This work has been carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. All of the studies were performed retrospectively by collecting and analyzing data from the patient records. This study had been approved by the Institutional Review Board of our hospital.

Design and case selection

For this retrospective, hospital based case-control study, subjects were recruited from patients attending the 3rd Internal Medicine of Pordenone General Hospital (Pordenone, Italy). The 3rd Internal Medicine is a tertiary referral center for liver diseases and diabetes mellitus. The case group consisted of patients with incident HCC admitted to the hospital. We then recruited two comparison groups: the first consisted of patients admitted for liver cirrhosis (LC); the second consisted of control individuals admitted for a wide spectrum of acute conditions other than diabetes mellitus and liver disease as primary diagnoses.

HCC group

The HCC group consisted of 465 (364 male, 101 female) consecutive patients with newly diagnosed HCC attending the 3rd Internal Medicine from January 1994 to June 2006. Of these, 398 (85.6%) were diagnosed by cytological or histological examination of hepatic focal lesions; the remaining 67 (14.4%) were diagnosed according to the following acknowledged criteria[28]: ultrasound examination (also by using micro-bubbles of sulfur hexafluoride as contrast dye in the last 3 years), α-fetoprotein (AFP) > 400 ng/mL, computerized tomography liver scan and/or magnetic resonance imaging. Based on the type of presentation, the HCC patients were subdivided into two groups: (1) 305 subjects from our surveillance program of HCC in cirrhotic patients (follow-up group: FU); and (2) 160 subjects presenting with symptomatic and advanced neoplastic disease of the liver (clinically overt: CO). The FU group included patients with a small single hepatic tumor who received the diagnosis within the HCC surveillance program in cirrhotic patients based on ultrasound examinations and AFP determinations every 3-6 mo; the CO group included cases with advanced, large size and symptomatic HCC at diagnosis. Clinical data, biochemical parameters and the antidiabetic treatment of patients were considered at the time of HCC diagnosis during the first admission to our Hospital.

In order to gain complete and accurate information on the time interval between DM2 onset and the diagnosis of HCC and to determine the levels of HbA1c testing before the HCC diagnosis, we reviewed, starting from October 1985, all medical documentation stored at the Diabetes Clinic.

Liver cirrhosis group

We enrolled 618 patients (450 male, 168 female) with LC, by selecting from 3560 cirrhotic patients treated at our Department subjects frequency-matched with the HCC cases, according to age (± 5 years), gender, body mass index (BMI), transaminases, serologic markers of HBV and HCV infections, alcohol consumption, time of hospital admission. The diagnosis of cirrhosis was performed either by hepatic biopsy or by ultrasound examination in the fasting state, showing the presence of splenomegaly, hypertrophy of the left or caudal lobes and surface irregularity or by transient elastography using the Fibroscan (Echosens, Paris) with a liver stiffness > 12.5 kPa[29]. LC patients were admitted to our Hospital for diagnosis, staging or therapy of LC. Clinical data, biochemical parameters and antidiabetic treatment were considered at the time of the first admission to our Hospital. According to Child's classification of cirrhosis, patients were classified as follows: class A: 55.5%; B: 24.3% and C: 20.2%. In cirrhotic patients, the presence of HCC was ruled out through ultrasound examinations, computed tomography or magnetic resonance imaging of the upper abdomen and AFP determination.

Control group

We enrolled 490 control subjects (385 male, 105 female) as follows: from 28740 inpatients of our hospital, we generated a pool of 7610 subjects with available tests as possible controls. Then we selected from them a frequency-matched subject for each HCC or LC patient, according to age (± 5 years), gender, BMI, time of hospital admission. Clinical data, biochemical parameters and therapeutic schedule of control subjects were considered at the time of the first admission to our Hospital. Subjects admitted for malignancies, alcohol-related disease, liver disease and DM2 as primary diagnoses were excluded from the study. The primary diagnoses of admission were: chronic heart failure (34.9%), hypertension (21.4%), chronic obstructive broncho-pneumopathies or pneumonia (16.5%), atrial fibrillation (7.8%), deep venous thrombosis (6.5%), fever of unknown origin (5.3%), benign tumors (4.1%), gastritis (3.5%). These subjects were assumed to represent our Region's general population as regards the prevalence of HCV, HBV infection, alcohol consumption and DM2. The prevalence of these parameters[30-33] in the free living population of the Pordenone area with an age range of 60-75 years and a similar age sub-sample of our control group is reported in Table 1.

Table 1 Mean prevalence of HCV infection, HBV infection, alcohol abuse and DM2 in the free living population of the Pordenone area and controls in this study

	General population (%)		Control group of the study (%)
	Global	60-75 yr age interval	
HCV positive	3.2	5.0	5.3
HBV positive	1.2	1.7	1.2
Alcohol abuse	4.5	5.0	4.7
DM2	4.8	12.4	12.6

HCV: Hepatitis C virus; HBV: Hepatitis B virus; DM2: Type 2 diabetes mellitus.

Methods

DM2 was diagnosed using the American Diabetes Association criteria[34]. Biochemical parameters were determined at the Pordenone Hospital central laboratory, using standardized and validated methods. Venous blood samples were taken in the morning after 12 h overnight fasting. Blood samples were available for 460 HCC cases and for all LC patients and controls. HbA1c was measured by high performance liquid chromatography (A1cHA-8160 Menarini, Italy)[35] for all diabetic subjects in our study. The nondiabetic range for our method of HbA1c testing was 4.0%-6.0% using a DCCT (Diabetes Control Complications Trial)-based assay[36]. For each subject we used the mean of 2 samples, taken on 2 consecutive days at the time of enrollment.

To assess the chronic diabetes controls in HCC patients with pre-existing DM2, we evaluated the individual HbA1c values tracked in the time before liver cancer diagnosis. Based on the records of the Diabetes Clinic, we determined the mean HbA1c levels of at least 3 tests/patient carried out approximately every 2 years before HCC clinical occurrence.

HBV surface antigen (HBsAg), anti-HBV surface antigen (anti-HBs), anti-HBV core antigen (anti-HBc), and hepatitis B e antigen (HBeAg) were determined using commercial assays (Abbott Diagnostic Division, Wiesbaden, Germany). Sera were also screened for antibodies against HCV (anti-HCV) using a third-generation microparticle enzyme immunoassay (AxSYM HCV version 3.0, Abbott Diagnostic Division). Positive samples were tested for anti-HCV using a third-generation line immunoassay (Immunogenetics, Gent, Belgium) and for serum HCV-RNA using the Roche Amplicor version 2.0 (Roche Molecular System, Pleasenton, CA, USA).

Information on consumption of alcoholic drinks was collected through a structured questionnaire which was returned by 455 HCC patients and by all LC and control subjects. Average alcohol content was estimated as 5% for beer, 12% for wine and 40% for spirits[37]. Alcohol abuse was defined as a daily consumption of over 30 g for males and over 20 g for females.

Statistical analysis

All the data were collected in a computerized database. Normality of the distributions of all continuous variables was tested by the Shapiro-Wilk test. The Student *t*-test and ANOVA were used to assess the statistical significance of

Table 2 Characteristics of patients in the HCC, LC and control groups *n* (%)

	HCC *n* = 465	LC *n* = 618	Controls *n* = 490	*P*
Sex, male	364 (78.3)	450 (72.8)	385 (78.6)	0.1706
Alcohol abuse	233 (51.2)	312 (50.6)	23 (4.7)	< 0.0001
DM2	145 (31.2)	144 (23.2)	62 (12.6)	< 0.0001
ALT ⩾ 53 IU/L	113 (24.3)	150 (24.3)	N/A	0.9911
HBV positive	39 (8.4)	39 (6.3)	N/A	0.1908
HCV positive	268 (57.6)	285 (46.1)	N/A	0.0002

P-value of χ^2 test. HCC: Hepatocellular carcinoma; LC: Liver cirrhosis; N/A: Not available.

Table 3 Association of DM2 with HCC: results of multivariate analyses using controls and LC subjects as comparison groups

	OR	95% CI	*P*
Control group			
Age ⩾ 65 yr	0.873	0.606-1.259	0.4675
BMI ⩾ 25 kg/m^2	1.048	0.769-1.426	0.7681
Alcohol abuse	22.069	13.749-35.425	< 0.0001
DM2	2.507	1.703-3.692	< 0.0001
LC group			
Age ⩾ 65 yr	2.457	1.855-3.252	< 0.0001
BMI ⩾ 25 kg/m^2	1.560	1.197-2.032	0.0010
HBV positive	2.372	1.363-4.127	0.0022
HCV positive	2.783	1.896-4.084	< 0.0001
Alcohol abuse	1.937	1.318-2.845	0.0008
ALT ⩾ 53 IU/L	0.919	0.672-1.256	0.5951
DM2	1.456	1.072-1.979	0.0162

BMI: Body mass index; OR: Odds ratio; 95% CI: 95% confidence interval; ALT: Alanine transaminase.

differences among groups for continuous variables with normal distribution; the Wilcoxon rank sum and Kruskal-Wallis tests were used for continuous variables with non-normal distribution. The χ^2 test was used to evaluate the significance of differences among groups for categorical variables. Multivariate logistic regression was used to assess the association between HCC and DM2, antidiabetic therapy, and glycemic control after adjusting for potentially confounding factors. Separate models were built considering LC patients and controls as the comparison groups. When assessing the association of DM2 with the risk of HCC using LC patients as the control group, we considered sex, age (⩾ 65 years *vs* < 65 years), BMI (⩾ 25 kg/m^2 *vs* < 25 kg/m^2), HBV and HCV infection, alcohol abuse, alanine transaminase (ALT) level (> 53 IU/L *vs* ⩽ 53 IU/L); when we used control subjects as the comparison group, potential confounders included only sex, age, BMI, and alcohol abuse. When evaluating which DM2 characteristics were associated with risk of HCC among diabetics, covariates included sex, age, BMI, HBV and HCV infection, alcohol abuse, ALT level, triglycerides, cholesterol, type of antidiabetic therapy (metformin, sulphonylureas, insulin), HbA1c levels (continuous and in categories using the following cut-offs: 6.5%, 7.5%, 8.5%), DM2 duration (continuous and using the following 3 cut-offs: 5, 10, 15 years) when we used LC patients as the comparison group and sex, age, BMI, HBV and HCV infection, alcohol abuse, ALT level, triglycerides, cholesterol, type of antidiabetic therapy, HbA1c levels, and DM2 duration when we used the controls. Among HCC cases, HbA1c levels at the time of cancer diagnosis were compared with an average of at least 3 tests carried out approximately every 2-3 years before the diagnosis of HCC. The Wilcoxon signed rank test and Spearman's correlation coefficient (*r*) were used to compare the paired differences and the kappa statistic to evaluate concordance of categories. Since at enrollment DM2 duration was either unknown or < 12 mo duration in 14% of diabetic subjects and 1-2 years duration in an additional 8%, in the study of the association between DM2 and HCC we performed a sensitivity analysis considering as non-diabetic subjects those whose diagnosis of DM2 occurred in the 12, 24, 60, and 120 mo before enrollment to allow for adequate induction time. For the same reason, we performed a sensitivity analysis excluding from the analyses regarding diabetes therapy and glycemic control, those patients with diagnosis of DM2 in the 12, 24, 60, and 120 mo before enrollment. All *P*-values < 0.05 were considered statistically significant. Analyses were conducted with SAS v9.1 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Demographic characteristics of the three groups studied, and exposures relevant to liver diseases are illustrated in Table 2. The prevalence of DM2 was significantly higher ($P < 0.0001$) in HCC patients (31.2%) than in the LC group (23.2%) and in controls (12.6%). Using control subjects as a comparison group, after adjusting for age, BMI and alcohol abuse, DM2 significantly increased the odds ratio (OR) of HCC by 2.5 times (Table 3). Using LC patients, after adjusting for sex, age, BMI, alcohol abuse, HBV and HCV infection, and ALT level, DM2 significantly increased the OR of HCC by 1.5 times (Table 3). Alcohol abuse was the strongest predictor of HCC using controls subjects as comparison; it also increased the risk of HCC, as did HBV and HCV infections by comparing HCC with LC patients.

Table 4 illustrates the levels of BMI, cholesterol, triglycerides, and HbA1c, in HCC, LC and control subjects with DM2. BMI, cholesterol and triglycerides were not different between the three groups of subjects studied. The HbA1c levels evaluated at the time of enrollment in the study were significantly higher in HCC patients (7.5%, with 8.1% ± 1.8% in FU and 6.3% ± 1.1% in CO subgroups $P < 0.0001$) than in controls (6.8%) and in LC patients (6.6%). Among HCC diabetic cases, current HbA1c levels were on average only 0.2 percentage points lower than the average value of 3 previous values that we assumed to be representative of glycemic control in the past years. Current and average past HbA1c levels were highly correlated ($r = 0.77$, $P < 0.0001$) and agreement between categories (using 6.5%, 7.5%, and 8.5% as the cut-offs) was moderate to good, as expressed by the weighted kap-

Table 4 Biochemical characteristics of diabetic HCC, LC patients and controls, DM2 duration and therapy (mean ± SD)

	HCC (n = 145)	LC (n = 144)	Controls (n = 62)	P
BMI (kg/m^2) (median)	25.6 ± 2.9 (26.0)	25.8 ± 3.8 (25.0)	25.1 ± 2.4 (25.3)	0.3175[a]
Cholesterol (mg/dL) (median)	155.4 ± 47.4 (152)	155.9 ± 37.7 (155)	160.6 ± 45.4 (173.0)	0.6013[a]
Triglycerides (mg/dL) (median)	110.6 ± 60.7 (94.5)	108.0 ± 45.5 (101.5)	111.5 ± 67.9 (95.0)	0.8060[a]
Fasting plasma glucose (mg/dL) (median)	126.7 ± 47.3 (110.5)	109.3 ± 32.9 (100.0)	98.0 ± 20.6 (95.0)	< 0.0001[a]
Current HbA1c (%) (median)	7.5 ± 1.8 (7.3)	6.6 ± 1.5 (6.4)	6.8 ± 1.5 (6.5)	0.0001[a]
Average past HbA1c (%) (range)	7.7 ± 1.5 (4-13.5)	N/A	N/A	
DM2 duration (mo) (median)	141.6 ± 81.0 (146)	135.4 ± 98.3 (117)	124.9 ± 100.5 (119)	0.2496[a]
Therapy, n (%)				< 0.0001[b]
Not reported	0 (0)	12 (8.3)	2 (3.2)	
Metformin	13 (9.0)	39 (27.1)	15 (24.2)	
Sulphonylureas	68 (46.9)	33 (22.9)	32 (51.6)	
Insulin	64 (44.1)	60 (41.7)	13 (21.0)	

[a]P of Kruskal-Wallis test; [b]P of Fisher's exact test. HbA1c: Glycated hemoglobin A1c.

pa 0.5456. Mean HbA1c levels were not different among HCC patients with or without HBV, HCV and alcohol abuse (data not shown).

Table 4 also shows the time interval between DM2 diagnosis and enrolment in the study. Duration of diabetes was not significantly different among the three groups of subjects and, on average, it was approximately 12 years. In addition, Table 4 displays the distribution of antidiabetic treatments in the three study groups. Treatment with metformin was less frequent among HCC patients than among the others; sulphonylureas were the most frequently used drugs among controls, whereas insulin was the most common treatment among HCC and LC patients.

At enrolment, DM2 had been diagnosed for more than 1 year in 86% of study subjects. Based on the records of our Diabetic Clinic, the mean duration of insulin treatment in HCC insulin-treated patients was 5 years; before insulin therapy, patients were treated only with diet. The mean period of treatment with the 2 antidiabetic oral agents was 10 years and before oral antidiabetic therapy patients had only dietary therapy.

Among diabetic subjects, the association of HbA1c levels, antidiabetic therapy, and DM2 duration with the risk of HCC is reported in Table 5. Both when using controls and when using LC as the comparison groups, after adjusting for the potential confounders shown in Table 5, therapy with metformin was associated with a strong and statistically significant reduction (> 80%) of the risk of HCC as compared with the use of sulphonylureas or insulin. In our HCC diabetic patients we found a significant 26%-50% increase in the HCC risk for each 1% increase in HbA1c level. Results did not significantly change in the sensitivity analysis after removing or moving from the diabetic to the non-diabetic category subjects whose diagnosis of diabetes occurred < 12, 24, 60, or 120 mo previously (data not shown).

Table 5 Association of antidiabetic therapy, metabolic control and DM2 duration with HCC among diabetic patients: results of multivariate analyses using controls and LC subjects as comparison groups

	OR[1]	95% CI	P
Control group			
Sex, male	1.860	0.636-5.441	0.2574
Age ≥ 65 yr	0.225	0.071-0.715	0.0115
BMI ≥ 25 kg/m^2	0.888	0.364-2.166	0.7936
Alcohol abuse	16.155	4.798-54.389	< 0.0001
Triglycerides (mg/dL, continuous)	1.001	0.994-1.008	0.8097
Cholesterol (mg/dL, continuous)	1.003	0.994-1.013	0.4890
Metformin *vs* sulphonylureas	0.149	0.039-0.507	0.0054
Insulin *vs* sulphonylureas	1.243	0.459-3.366	0.6686
DM2 duration (mo, continuous)	1.001	0.996-1.005	0.6740
HbA1c % (continuous)	1.265	0.943-1.699	0.1172
LC group			
Sex, male	0.120	0.0051-0.278	< 0.0001
Age ≥ 65 yr	1.508	0.719-3.165	0.2774
BMI ≥ 25 kg/m^2	1.473	0.716-3.029	0.2928
HBV+	3.535	0.428-29.184	0.2410
HCV+	2.870	1.129-7.293	0.0267
Alcohol abuse	1.455	0.567-3.732	0.4351
ALT ≥ 53 IU/L	0.808	0.394-1.675	0.5606
Triglycerides (mg/dL, continuous)	1.002	0.995-1.008	0.6366
Cholesterol (mg/dL, continuous)	1.003	0.995-1.011	0.4012
Metformin *vs* sulphonylureas	0.163	0.057-0.462	0.0006
Insulin *vs* sulphonylureas	0.428	0.203-0.901	0.0255
DM2 duration (mo, continuous)	1.001	0.997-1.005	0.6723
HbA1c % (continuous)	1.508	1.197-1.899	0.0005

[1]Patients with unknown therapy not included in analysis.

DISCUSSION

The results of our study show that chronic poor glycemic control, evaluated by HbA1c testing, is positively associated with risk of HCC in diabetic patients. In addition, therapy with metformin was associated with a strong and statistically significant reduction in the risk of HCC as compared with the use of sulphonylureas and insulin therapy.

Several studies have reported the relationship between DM2 and carcinogenesis in the liver[4-9], but, as far as we know, there are no investigations on the relationship between glycemic control and HCC risk in DM2 patients. In our study, we found that the prevalence of DM2 patients was significantly higher in HCC patients whereas in the LC group it was intermediate between those of HCC cases and controls. After adjusting for potential con-

founders, in our HCC patients DM2 was associated with an increased risk of HCC. The HbA1c mean levels were significantly higher in diabetic HCC patients than in LC and controls but not different between HCC patients with or without HBV and HCV infection or alcohol abuse. In HCC patients (above all in the FU subgroup), the HbA1c values were significantly higher and the mean levels during previous years of diabetic life were similar to those recognized at HCC occurrence. Multivariate analysis showed that high levels of HbA1c among diabetic HCC patients were associated with a significantly increased risk of liver cancer.

Because of IR, patients with DM2 have a long-term exposure to increased circulating insulin levels. It is well known that insulin stimulates cellular mitosis by direct action[14], and indirectly by stimulating the insulin-like growth factor-1 intracellular pathway, a major mitogenic and anti-apoptotic effector in carcinogenesis[15]. Sulphonylurea treatment increases insulin secretion and its circulating levels: this effect is detrimental in terms of weight gain and insulin levels[38]. In contrast, metformin treatment can ameliorate IR and reduce weight gain. The ability of metformin to reduce the insulin plasma levels and to activate cellular AMP-activated protein kinase (AMPK) represents, respectively, its direct and indirect proposed anti-oncogenic mechanisms. In fact, metformin not only lowers blood glucose and insulin levels but, through AMPK activation, also attenuates the *in vitro* response of cancer cells to insulin[39,40].

Several *in vivo* studies showed that cancer risk was lower in patients exposed to metformin than in unexposed patients[41,42]. Metformin has also been shown to be potentially beneficial in patients with specific types of cancer. For example, DM2 patients receiving neoadjuvant chemotherapy for breast cancer as well as metformin were more likely to have a complete remission than patients not receiving metformin[43]. In 2 studies patients receiving metformin seemed to have a lower incidence of prostate and pancreas cancer[44,45]. Furthermore, in the ZODIAC study[46], metformin use was associated with lower cancer mortality when compared to non metformin use.

In diabetic patients, good glycemic control prevents the onset and progression of acute and long-term diabetes-related complications[47]; however, it is presently unknown if poor metabolic control of diabetes could enhance or accelerate liver carcinogenesis in patients with CLD and DM2. Furthermore, it is not known whether an optimized control of diabetes can reduce the risk or delay the development of liver cancer. In the European Prospective Investigation into Cancer Study, a 1% increase in HbA1c levels was associated with a 1.3-fold increase in the risk of colorectal cancer[48]; recently it was reported that poor glycemic control is a predictor of clinically aggressive course for the colorectal cancer[49]. HbA1c reflects average glycemia over 3 mo; in our HCC patients, particularly in FU cases, the mean levels of HbA1c were above the recommended target level of 7%; HbA1c was significantly higher in HCC than in controls and LC patients, in which we found mean levels below 7%. Nonetheless it must be emphasized that the HbA1c levels observed in the three groups of subjects in this study were consistent with those seen in the vast majority of Italian diabetic patients[10].

It is known that diabetes is associated with more advanced lesions and poor outcomes in patient with HCC[50] but the observation that DM2 precedes the diagnosis of HCC in the majority of our cases, suggests that glucose intolerance and glycemic control are an outstanding feature of the hepatic tumor that may not always be related to the liver cancer.

This was a retrospective, hospital-based study drawn from a clinical series and not from the community: therefore the results may not be representative of those of the general population. There is no evidence that successful treatment for glucose intolerance can reduce hepatic carcinogenesis: therefore only a prospective study is required to demonstrate that optimized glycemic control can modify the risk of HCC in DM2 patients with CLD. However, this is the first study on the relationship between different antidiabetic strategies and the presence of HCC. In our diabetic HCC patients, HbA1c test results are available for years before HCC diagnosis, so a reliable assessment of prior glycemic control was possible. Our study was a single center investigation and all patients of the three groups studied were directly diagnosed and followed-up by us; all biochemical parameters were determined in a single laboratory. The study population was large. The controls, even though recruited from inpatients, were representative of the general population of our Region as regards the prevalence of HCV and HBV infection, alcohol consumption and diabetes mellitus.

To distinguish the temporal relationship between exposure and outcomes, due to the complex and reciprocal relationships between DM2, LC and HCC, we conducted a study on a large group of HCC patients comparing them with both a control group with no liver diseases and a group with LC, that represents the majority of patients with the clinical underlying cause of HCC. To have complete and accurate information on the time interval between the onset of DM2 and the diagnosis of HCC, we reviewed all medical documentation kept, from a period of 9 years before the enrollment of the first patient in the study, at our Diabetes Clinic. All our diabetic patients were on monotherapy of antidiabetic drugs and the selection of the antidiabetic therapy was based on the physician's clinical choice. In particular, in HCC and LC groups, diabetic patients treated with different antidiabetic oral agents had similar basic features as regards alcohol consumption, and hepatic and renal function. To make sure that a temporal relationship existed between diabetes and HCC, a sensitivity analysis was conducted excluding or reclassifying patients with recent diabetes diagnosis, but the results did not change.

Our study confirms that DM2 is associated with an increased risk of HCC. It also shows that in diabetic HCC patients metformin treatment is associated with a strong and statistically significant reduction (> 80%) in the risk of HCC compared with the use of sulphonylureas or

insulin. In diabetic patients with CLD, chronic poor glycemic control, evaluated by HbA1c testing, significantly increases the risk of HCC by 26%-50% for each 1% increase in HbA1c level. It has to be emphasized that in diabetic patients with CLD, it is important not only to attain an optimized glycemic control in order to prevent HCC, but also antidiabetic strategies may have an important effect on the relationship between DM2 and solid tumors.

COMMENTS

Background

In recent years, several investigations clearly indicated that type 2 diabetes mellitus (DM2) is a risk factor for hepatocellular carcinoma (HCC). The aims of the present study were to explore this association and to assess the relationships of antidiabetic therapy and glycemic control with the risk of HCC in DM2 patients with chronic liver disease.

Research frontiers

At the present time the relationship between DM2 and solid cancers is under intensive investigation. It remains unknown if DM2 has a direct carcinogenic effect on the liver and on other parts of the human body.

Innovations and breakthroughs

This study found that DM2 precedes HCC in the majority of patients. The cancer risk seems to be increased by chronic poor glycemic control. Metformin treatment is associated with a strong and statistically significant reduction (> 80%) in the risk of HCC compared with the use of sulphonylureas or insulin.

Applications

The study indicated that in diabetic patients with chronic liver disease it is important not only to attain an optimized glycemic control in order to prevent HCC, but also that antidiabetic strategies may have an important effect on the association between DM2 and solid tumors.

Peer review

This is a potentially important paper that continues to evaluate the link between diabetes and the risk of HCC. Its strength is in its numbers despite being from one institution.

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BRIEF ARTICLE

Helicobacter pylori in dental plaque and stomach of patients from Northern Brazil

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Abstract

AIM: To establish whether virulence factor genes *vacA* and *cagA* are present in *Helicobacter pylori* (*H. pylori*) retrieved from gastric mucosa and dental plaque in patients with dyspepsia.

METHODS: Cumulative dental plaque specimens and gastric biopsies were submitted to histological examination, rapid urease test and polymerase chain reaction (PCR) assays to detect the presence of *cagA* and *vacA* polymorphisms.

RESULTS: Detection of *H. pylori* from dental plaque and gastric biopsy samples was greater by PCR compared to histological examination and the rapid urease test. DNA from *H. pylori* was detected in 96% of gastric mucosa samples and in 72% of dental plaque samples. Sixty-three (89%) of 71 dental plaque samples that were *H. pylori*-positive also exhibited identical *vacA* and *cagA* genotypes in gastric mucosa. The most common genotype was *vacAs1bm1* and *cagA* positive, either in dental plaque or gastric mucosa. These virulent *H. pylori* isolates were involved in the severity of clinical outcome.

CONCLUSION: These pathogenic strains were found simultaneously in dental plaque and gastric mucosa, which suggests that gastric infection is correlated with the presence of *H. pylori* in the mouth.

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Key words: *Helicobacter pylori*; Gastric mucosa; Dental plaque; *cagA*; *vacA*

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INTRODUCTION

Helicobacter pylori (*H. pylori*) gastric infection is considered one of the most common human infections[1]. It occurs in half of the world's population[2,3] and is the most common cause of adenocarcinoma of the distal stomach[4].

The risk in developing gastric cancer is believed to be related to differences among *H. pylori* strains and the inflammatory responses mediated by host genetic factors[3]. Therefore, it is presumed that severe gastric diseases are seen more often in patients who have been chronically infected with *H. pylori* isolates that bear both the *cagA* and *vacA* s1/m1 genes[1,5,6].

H. pylori is known to be acquired early in life[7,8], and is likely to be transmitted from person to person. The transmission is supported by crowded living conditions, accompanied by poor hygiene and intra-familial clustering. Nevertheless, the exact manner of transmission is not completely understood[4,9,10].

The accepted evidence is that the *H. pylori* strains reach the stomach by ingestion through the mouth, and because of its non-invasive nature, the stomach is the definitive site for colonization[10]. Evidence of transmission *via* the oral route includes the high prevalence of *H. pylori* among African children whose mothers premasticate their food before offering it to them[11], and to the higher prevalence of the infection in people who share chopsticks[12].

The presence of *H. pylori* in the oral cavity was first reported in 1989 when the bacterium was cultured from dental plaque of a patient with gastric disease associated with *H. pylori* infection[13].

Dental plaque is a biofilm that is formed by a microbial community of multiple species and represents a strategy to allow survival and evolution of microbial co-aggregation in a dynamic equilibrium, in a favorable environment with selective advantages[14,15]. Hence, bacterial biofilms are considered as sanctuaries, due to being protected from host defense mechanisms and antibiotic therapy. Additionally, antibiotic resistance is increased due to horizontal gene transfer[14,15].

The purpose of this study was to evaluate the prevalence of *H. pylori* isolates, in relation to *vacA* and *cagA* genotypes, in dental plaque and gastric antral biopsy samples in dyspeptic patients from a North Brazilian population.

MATERIALS AND METHODS

Patients and sampling

We studied the presence of *H. pylori* in the stomach and dental plaque in 99 adult patients (69 women and 30 men; mean age: 37 years, age range: 17-59), who underwent upper gastrointestinal endoscopy due to gastric problems. Most of them were of lower socioeconomic status and lived in the state of Pará, Brazil.

None of the patients had received antimicrobial drugs, H2-receptor antagonists, acid pump inhibitors, non-steroidal anti-inflammatory drugs, or any medication for at least 60 d before sampling, to avoid interference with *H. pylori* detection methods. During endoscopy, three antral gastric biopsy sections were taken from the stomach of each patient. One biopsy was analyzed by molecular methods, and the other sections were also analyzed with histological methods and the rapid urease test. Before endoscopic examination, cumulative dental plaque samples were collected by scraping tooth surfaces with sterile curettes and transferred into tubes that contained physiological saline solution. Dental plaque samples were frozen immediately and stored at -20℃ until required for DNA extraction. This study was approved by the Ethics Committee of Hospital Universitário João de Barros Barreto, Belém, PA, Brazil. All patients gave their informed consent to participate in the study.

Histological evaluation

The biopsy specimens were fixed in 10% buffered formalin solution, embedded in paraffin, cut into sequential 0.4-μm sections, and stained with hematoxylin and eosin (H&E). The presence of *H. pylori* in the sections was determined by using a modified Gram staining protocol and taking into consideration its morphological characteristics: curved and spiral form and intense purple coloring. The histological parameters were graded (0-3) using the criteria described in the update Sydney classification system[16] for analysis of chronic inflammation, polymorphonuclear activity and intestinal metaplasia.

H. pylori detection by the rapid urease test in the stomach and dental plaque

Additional fresh biopsies as well as dental plaque samples were placed in the gel of the CLOtest as recommended by the manufacturer (Uretest; Renylab, Barbacena, MG, Brazil). After 1 h, the CLOtest was inspected for a change in color. Yellow was interpreted as negative, indicating absence of urease. Red or pink was interpreted as positive. For comparison, a similar reading of each biopsy was also performed after 3 and 24 h. Results of the rapid urease test were compared with polymerase chain reaction (PCR) results and histological examination.

DNA isolation

Total DNA was extracted from frozen gastric biopsy and dental plaque specimens using the following procedure[17]: 10 μL proteinase K (Amresco, Cleveland, OH, USA) and 300 μL lysis buffer (200 mmol/L Tris-HCl, 25 mmol/L EDTA, 300 mmol/L NaCl, 1.2% SDS) were added to the biopsy or dental plaque pellets specimens. The mixture was incubated at 55℃ for 12 h. The lysate was extracted with an equal volume of phenol-chloroform, precipitated with isopropanol, and washed with 70% ethanol. The DNA pellet of each sample was dried and resuspended in 200 mL sterile distilled water. DNA extracts were stored at -20℃. Extensive care was taken to avoid contamination during all steps of collecting and preparing the samples.

PCR amplification and detection of amplified DNA products

PCR amplification for detection of *H. pylori* DNA in dental plaque and gastric mucosa was performed as described previously[18]. Briefly, one set of primers (p1-F and p2-R) that amplifies a 26-kDa antigen gene (fragment of 298 bp) present in all strains of *H. pylori* was used to detect bacteria[18]. The previously described F1-F and B1-R primers[19] were used to detect *cagA* gene. The amplification of *vacA* gene was performed by PCR with oligonucleotide primers described by Atherton *et al*[1], *vacA* signal (*vacA s1a, s1b* or *s2,* primers SS1- F, SS3-F, SS2-F/VA1-R, respectively) sequences and middle regions (*vacA m1* or *m2,* primers VA3-F/VA3-R and VA4-F/VA4-R).

All PCR mixtures were prepared in a volume of 25 μL that contained 0.5 nmol/L each primer; 1 × PCR buffer; 1.5 nmol/L $MgCl_2$, sterilized water, 0.2 nmol/L deoxynucleoside, 1.25 μL *Taq* DNA polymerase Platinum (Invitrogen Life Technologies, São Paulo, Brazil), and 2 μL DNA sample. The mixtures were placed in a thermal cycler. PCR amplification was performed under the following conditions: initial denaturation at 95℃ for 2 min, followed by 35 cycles of denaturation at 95℃ for 1 min, annealing and extension for 1 min, and final extension at 72℃ for 10 min. Annealing temperatures were set at 58℃ for primers VA3-F/VA3-R, VA4-F/VA4-R, F1/B1, and at 63℃ for SS1- F, SS3-F, SS2-F/VA1-R. Negative (sterile water) and positive (positive strain) controls were used in all reactions. PCR products were visualized by electrophoresis in 2% agarose gel, stained with ethidium bromide, and examined under UV illumination.

Statistical analysis

Data were analyzed with Biostat 4.0 version software[20]. Mann-Whitney and G tests were used to assess the association between *H. pylori* isolates in dental plaque and stomach and the distribution of *vacA* and *cagA* genotypes in both these specimens. Differences were considered statistically significant for P values less than 0.05.

RESULTS

Identification by PCR of *H. pylori* DNA in gastric biopsies and dental plaque

H. pylori was found in gastric biopsies of 96% (95/99) of the patients and the detected frequency of this organism in dental plaque was 72% (71/99). Both were detected by PCR, but none of the four patients that lacked *H. pylori* in the stomach had this organism in dental plaque. This difference was statistically significant, which indicated that the detection frequency of *H. pylori* in dental plaque was lower than in the stomach ($P < 0.001$).

Relationship of *H. pylori* DNA in dental plaque and stomach with age and sex

In the relative distributions of age and sex of the patients studied, no significant difference was observed between the groups with or without positive DNA for *H. pylori* simultaneously in the gastric mucosa and dental plaque. Nine of 30 (30%) men and 16 of 69 (27.5%) women were *H. pylori*-negative in dental plaque. Among these, 19 of 62 individuals belonged to the 17-39 years age group and eight of 33 to the 40-59 years group. Furthermore, three individuals in the first group and one in the second were *H. pylori*-negative in dental plaque and gastric mucosa.

Relationship of *H. pylori* DNA in dental plaque and stomach with clinical and histopathological parameters

All patients presented an inflammatory process in the gastric mucosa, with a diagnosis of functional dyspepsia. Ulcers or premalignant lesions, such as intestinal metaplasia, which were considered as lesions of higher severity, occurred in 17% (17/99) of the patients. Among these, 82.4% of cases harbored *H. pylori* in the stomach and dental plaque.

The genotypic frequencies of *vacA* and *cagA* of the positive *H. pylori* samples in the gastric biopsies and dental plaque DNA samples in relation to the endoscopic clinical diagnosis and histopathological findings are shown in Table 1. The *vacAs1m1/cagA*-positive genotype was found in 51 of 95 *H. pylori* gastric biopsies, which comprised 44 gastritis specimens and seven peptic ulcer samples, with 46 patients being *H. pylori*-positive in dental plaque DNA samples.

The presence of *H. pylori* mixed strains with *vacA s1*, *s2*, *m1*, *m2* and *cagA*-positive multiple genotypes was detected in 12 of 95 gastric biopsies of patients with gastritis and their corresponding dental plaque DNA samples.

The *vacAs2m2/cagA*-positive genotype was observed in four of 95 *H. pylori* gastric biopsies, which included one patient with peptic ulcer and three with chronic gastritis. The *vacAs2m2/cagA*-negative genotype was found in 28 of 95 *H. pylori* isolates from 27 gastritis specimens and one peptic ulcer sample, with 13 patients being simultaneously *H. pylori* positive in dental plaque DNA samples.

When comparing all these genotypes, there was 66% (63/95) agreement between *H. pylori* biopsy isolates and their corresponding dental plaque DNA samples. In addition, seven of nine patients with peptic ulcer and eight of 86 with chronic gastritis and diagnosed with intestinal metaplasia or gastric atrophy expressed *H. pylori* isolated with *vacAs1m1*- and *cagA*-positive genotypes, which indicated more severe gastritis and peptic ulceration (Table 1). In the same way, in relation to the histopathological data of these patients, a significant association has been observed between the presence of a bacterial virulence genotype and the high levels of inflammation and the neutrophilic activity, as well as an increased risk of developing atrophy and intestinal metaplasia in the gastric mucosa.

Detection of *H. pylori* in dental plaque and gastric biopsies by different diagnostic tests

In addition to the evaluation of *H. pylori* by PCR assay, bacteria in the stomach were also detected by the histological examination and the rapid urease test. However, the analysis of urease test indicated gastric colonization by *H. pylori* in only 49% of the patients. Similarly, evidence of *H. pylori* was detected in 52% of dental plaque speci-

Table 1 Frequency of *vacA* and *cagA* genotypes in patients positive for *H. pylori* DNA in dental plaque and stomach

H. pylori genotypes					Histopathological parameters[a]						Endoscopic diagnosis[b]		Total
Stomach		Dental plaque			DI		NA		IM	*H. pylori*	PU	G (+H)	
vacA	*cagA*	C	D	N	1	2/3	1	2/3	+	+			
s1m1	+	42	4	5	21	30	26	25	8	22	7	34/(10)	51
s1m1, s2m2	+	12	-	-	5	7	6	6	3	5	-	10/(2)	12
s2m2	+	-	-	4	2	2	2	2	1	1	1	2/(1)	4
s2m2	-	9	4	15	25	3	22	6	-	11	1	22/(5)	28
Total		63	8	24	53	42	56	39	12	39	9	72/(14)	95

DI: Degree of inflammation; NA: Neutrophilic activity (histopathological scores: 1, light; 2, moderate; 3, intense); IM: Intestinal metaplasia; *H. pylori*: *Helicobacter pylori*; G: Gastritis; H: Hiatus hernia; PU: Peptic ulcer; N: Polymerase chain reaction (PCR) negative for *H. pylori* in dental plaque; C: Identical genotypes in the stomach and dental plaque; D: Distinct genotypes in the stomach and dental plaque. [a]G tests (Yates) = 16.83, $P < 0.001$ (DI: *s1m1 cagA* + *x s2m2 cagA*-); G tests (Yates) = 4.86, $P = 0.027$ (NA: *s1m1 cagA* + *x s2m2 cagA*-); [b]G tests (Yates) = 4.36, $P = 0.037$ (disease: *s1m1 cagA* + *x s2m2 cagA*-).

Table 2 Results of the different diagnostic tests for *H. pylori* detection in gastric biopsies and dental plaque samples *n* (%)

Techniques used in the specimens	*H. pylori*		Total
	Positive	Negative	
Dental plaque[a]			
PCR	71 (72)	28 (28)	99
Urease	48 (52)	45 (48)	93
Stomach[b]			
PCR	95 (96)	4 (4)	99
Histological[1]	39 (48)	43 (52)	82
Urease	47 (49)	49 (51)	96

[1]31 of 39 were also urease positive; [a]G tests (Yates) = 7.39, $P = 0.006$; [b]G tests (Williams) = 76.82, $P = 0.0001$ (PCR/Histological/Urease); G tests (Yates) = 57.14, $P = 0.0001$ (PCR/Histological); G tests (Yates) = 58.48, $P = 0.001$ (PCR/Urease); G tests (Yates) = 0.001, $P = 0.97$ (Urease/Histological).

Table 3 Comparison of *H. pylori* isolates in the stomach and dental plaque, by PCR assay *n* (%)

Genotypes		Stomach	Dental plaque
vacA	*cagA*		
s1am1	+	16 (17)	16 (22)
s1bm1	+	35 (37)	26 (37)
s2m2	+	4 (4)	-
s1bm1/s2m2 (mixed infection)	+	12 (13)	16 (22)
s2m2	-	28 (29)	9 (13)
s1bm2/s2m2 (mixed infection)	-	-	4 (6)
		95 (100)	71 (100)

Mann-Whitney test: $U = 15.00$, $Z(U) = 0.48$, $P = 0.63$.

mens, using the rapid urease test (Table 2). There were significant differences among *H. pylori* positivity detected by PCR and the other methods used ($P < 0.001$).

Comparison of *H. pylori* vacA and cagA genotypes in dental plaque and gastric mucosa samples

Overall, 63 (89%) of 71 dental plaque samples, which were positive for the presence of the bacteria, revealed identical *H pylori* virulence factors in relation to *vacA* and *cagA* genotypes in the gastric mucosa. *H. pylori* isolates found in 71% (67/95) of gastric mucosa specimens were *cagA*-positive and 82% (58/71) were also *cagA*-positive for dental plaque, with significant correlations among the prevalence of *H. pylori cagA*-positive isolates in the stomach and dental plaque samples (Table 3).

In relation to the *vacA* gene, the *s1m1* genotype had a higher frequency, with 54% (51/95) of gastric biopsies and 59% (42/71) of dental plaque samples However, the *vacAs2m2* subtype combination was found among the patients that harbored *H. pylori* in 34% (32/95) of the stomach and in 13% (9/71) of the dental plaque samples, whereas *vacAs1bm1/s2m2* genotypes were detected in 13% (12/95) of gastric biopsies and 22% (16/71) of dental plaque samples (Table 3). The most common genotype was *vacAs1bm1* and *cagA*-positive, either in dental plaque (37%) or gastric mucosa (37%).

DISCUSSION

H. pylori infections of the stomach are common worldwide. These bacteria are also found in dental plaque, where the presence of this organism may be very low and the numbers of microorganisms appear to vary from one site to another within the mouth[21].

The present study detected DNA from *H. pylori* in 95% of clinical gastric biopsy samples. Likewise, as described in previous studies[6,22,23], the occurrence is very high in this community. In fact, *H. pylori* infection is influenced by precarious sanitation and low socioeconomic level, so that the prevalence of *H. pylori* infected subjects in Brazil can reach over 80%[24,25]. Furthermore, 72% of subjects with stomach infection showed a concomitant presence of *H. pylori* in dental plaque by PCR assay.

The high detection rate for *H. pylori* DNA in the dental plaque is sufficiently significant to support the assumption that *H. pylori* reaches the oral cavity by reflux[21], assisted by the finding that no positive case was found in dental plaque that was negative in gastric mucosa. This result is especially important because, in our sample, only untreated patients were selected, since treatment would likely eradicate the bacteria from the stomach but rarely from dental plaque, due to known resistance to systemic therapy in dental plaque, as has been reported by Gurbuz *et al*[26].

Similar results have been reported by Banatvala *et al*[13], who have verified a 63% correlation between *H. pylori* in the stomach and in dental plaque samples in patients from London. Also, Song *et al*[27] have found *H. pylori* in 97% (41/42) of dental plaque samples obtained in a German population.

Other studies have suggested a positive association between dental plaque and gastric *H. pylori*[28-32], but some others have failed to find *H. pylori* in oral samples[33-35]. In support of the former studies, our results indicate that the frequency of *H. pylori* in dental plaque is related to the existence of gastric infection.

In the patients studied, no significant difference was observed in the rate of *H. pylori* DNA in dental plaque and gastric mucosa of the diverse age groups. These results are similar to the infection patterns in developing countries[7,36]. A similar result was found in relation to sex, which indicated that there was no relation between sex ratio and *H. pylori* infection in the stomach and dental plaque DNA samples. However, Berroteran *et al*[34] have detected a higher level of *H. pylori* in gastric samples from women than from men, but in dental plaque, this difference was not observed.

On the other hand, these data indicate that, in the strains that harbor *vacAs1m1, cagA* genotypes are involved in the severity of gastritis and the final outcome of *H. pylori* infection. In addition, *H. pylori* isolates with *cagA* and *vacAs1m1* genotype are associated with an increased risk of developing distal gastric adenocarcinoma[37].

In the present study, different methods were used for detecting *H. pylori* in dental plaque and gastric mucosa. In this regard, the PCR assay has been demonstrated to be more efficient for the detection of *H. pylori* in clinical specimens, compared to the rapid urease test in dental plaque and gastric biopsies, as well as to the histopathological examination of the gastric mucosa. This result is supported by other studies[27,30,34,38].

Dönmez-Altuntaş and Güven[39] have detected higher specificity and sensitivity of the PCR assay in relation to the urease test, with 93.8% *vs* 65.6% of DNA from *H. pylori* being positive in both tests respectively. Zsikla *et al*[40] have reported that *H. pylori* DNA could be detected by PCR in 20.8% of biopsies with chronic gastritis but without histologically detectable bacteria. These results agree directly with our findings. Despite this, one of the critical points of the studies using PCR is the absence of uniformity of results, due to the establishment of distinct laboratory procedures[27,41-43]. Controlled trials with robust methodology are required to prove if there is any clinical or epidemiological relevance to the colonization of this bacterium in the oral cavity.

The molecular characterization of the *H. pylori* virulence factors, *cagA* and *vacA*, has revealed the existence of indistinguishable genotypes from oral cavity and gastric mucosa samples of the same patient[44]. Our results showed that, in 89% of the 71 cases in which the dental plaque samples were positive for bacteria, the vacA and cagA genotypes were similar to those in the gastric mucosa (Table 3). Thus, these data suggest that similar types of *H. pylori* isolates, in relation to *vacA* and *cagA* genotypes, are present in the mouth and stomach.

We found that the most common genotype was positive for *vacAs1bm1* and *cagA*, either in dental plaque or gastric mucosa (Table 3). In addition, the *H. pylori* isolates that were *vacAs1m1*/*cagA*-positive were detected in dental plaque at a higher percentage than the *vacAs2m2*/*cagA*-negative genotype. It seems that *H. pylori* in dental plaque is related more to severe gastrointestinal diseases, such as gastroesophageal reflux, which could facilitate oral colonization by *H. pylori* with a *vacAs1m1*/*cagA*-positive genotype.

In this context, several investigators have reported that reinfection is caused by the strains in the oral cavity, because they have isolated identical strains of *H. pylori* in the oral cavity and stomach[32,45-49]. Therefore, it seems necessary to apply additional molecular tests in the dental plaque to assist the diagnosis and therapy of gastric infection.

Finally, based on our findings we cannot exclude the possibility that transient colonization of the mouth with *H. pylori* may occur by reflux activities within symptomatic patients, which leads to the spread these organisms by direct transmission from person to person. In this way, it is a risk factor for the occurrence of diseases in the oral cavity and the stomach.

COMMENTS

Background

Infection by *Helicobacter pylori* (*H. pylori*) remains one of the most prevalent chronic bacterial diseases worldwide, with a distribution related to the degree of economic development in each country. The organism is transmitted from person to person, and some researchers have suggested that the oral cavity is an important reservoir of *H. pylori* besides the stomach. Although the oral cavity may be a source of transmission, it is unknown whether the mouth could be a common source for reinfection of the stomach after treatment.

Research frontiers

The variability of clinical manifestations in the population could be related to an interaction between the bacterial virulence factors and genetic susceptibility of the host. *H. pylori* strains that have *cagA*-positive and *vacAs1/m1* genotypes are associated with an intense inflammatory response in the stomach, which leads to peptic ulcer and other severe gastric diseases. For this reason, there is an increasing interest in generating different non-invasive diagnostic tests that allow the epidemiological and clinical study of *H. pylori* infection.

Innovations and breakthroughs

In this study, dental plaque and gastric biopsies were submitted to rapid urease test and polymerase chain reaction (PCR) assays to detect *H. pylori* and the virulence factors, *cagA* and *vacA* genotypes, respectively. In addition, gastric biopsies were evaluated by histological techniques. It was found a high correlation between the most pathogenic strains (*vacAs1m1/cagA*-positive) in dental plaque and gastric mucosa. It seems that the severity of gastrointestinal diseases, such as gastroesophageal reflux, could be facilitated by oral colonization by *H. pylori*.

Applications

The simultaneous presence of the *H. pylori* isolates with similar *cagA* and *vacA* genotypes in dental plaque and gastric mucosa was detected in a high percentage in patients with chronic gastritis and peptic ulcer, which suggests the necessity to apply additional molecular tests to dental plaque to assist with the diagnosis and therapy of gastric infection. This will add to the available knowledge about the pathogenetic relevance of *H. pylori* in the oral cavity.

Terminology

Bacterial virulence factors are expressed by the vacuolating cytotoxin gene (*vacA*) and cytotoxin-associated gene (*cagA*).

Peer review

The authors studied by means of PCR whether *H. pylori*-positive subjects also had the bacterium in the dental plaque. They found that the vast majority of patients with stomach colonization by *H. pylori* also had oral bacterial infection, thus lending further support to the concept that oral to oral transmission of *H. pylori* is highly possible. The manuscript is well written.

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BRIEF ARTICLE

Predisposing factors and surgical outcome of complicated liver hydatid cysts

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Abstract

AIM: To evaluate the predisposing factors for peritoneal perforation and intrabiliary rupture and the effects of these complications on surgical outcome in liver hydatid disease.

METHODS: A total of 372 patients with liver hydatid cysts who had undergone surgical treatment were evaluated retrospectively. Twenty eight patients with peritoneal perforation, 93 patients with spontaneous intrabiliary perforation, and 251 patients with noncomplicated hydatid cysts were treated in our clinics.

RESULTS: When the predisposing factors for complications were evaluated, younger age, superficial position, and larger cyst dimensions ($P < 0.05$; range, 0.001-0.017) increased peritoneal perforation rates. It was shown that older age increased cyst dimensions, and presence of multiple and bilobar cysts increased intrabiliary rupture rates ($P < 0.05$; range, 0.001-0.028). Partial pericystectomy and drainage was the most frequent surgical procedure in all groups (71.6%). The incidence of postoperative complications in the peritoneal perforated group, in the intrabiliary ruptured group, and in the noncomplicated group was 25%, 16.1% and 5.5%, respectively. When compared, complication rates were significantly different ($P = 0.002$). When length of hospital stay was compared, there was no significant difference between the groups ($P > 0.05$). The overall recurrence rate was 3.8% (14 patients), but there was not any statistical difference among the patient groups ($P = 0.13$). The early postoperative mortality rate was 1.1%.

CONCLUSION: In peritoneally perforated and intrabiliary ruptured cases, the most important steps are irrigation of the peritoneal cavity and clearance of the cystic material from the biliary tree.

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Key words: Complicated liver hydatid cysts; Predisposing factors; Surgical treatment; Surgical outcome

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INTRODUCTION

Echinococcosis is a zoonosis transmitted by dogs that

accidentally infects humans. Both sheep and humans are intermediate hosts. Developing countries with poor hygiene where sheep and cattle are raised are high-risk areas for acquiring cystic echinococcosis. Endemic regions of human cystic disease include South America; the Mediterranean region, including North Africa, Spain, Portugal, and Turkey; the Middle East; central Asia; and many regions in China[1,2]. The prevalence of the disease increased in Europe and North America in recent years. In the United States and Europe, most infections are seen in immigrants from endemic areas[3].

In humans, 50% to 75% of hydatid cysts occur in the liver, 25% are found in the lungs, and 5% to 10% are distributed along the arterial system[4]. The majority of the patients have single-organ involvement (87%) and a solitary cyst (72%) at the right part of the liver[5]. If the cyst ruptures, scolices can grow in the peritoneum, pleura, bronchial tree, and bile ducts[5-7]. Cyst sizes range from 1 to 30 cm, and cyst growth varies from 1 to 30 mm per year[6,8].

The clinical picture can be severe, and complications may occur, making the already difficult treatment even more so[9,10]. Complications are observed in one third of patients with liver hydatid cysts. In large series, incidences of peritoneal perforation were reported at between 10% and 16%[11,12], and communications between the cyst and the biliary tree are seen in 9% to 25% of cases[13-15]. Without treatment, cysts grow and eventually may form fistulas into the peritoneal cavity or intrabiliary rupture, requiring emergency surgery[2,9,10]. Operative treatments vary from complete resection to minimal invasive procedures, but the ideal treatment is still controversial[16,17]. The choice of therapy depends on several factors: number and localization of the cysts, surgeon expertise, and presence of complications.

Only a few studies have reported the management and outcome of patients with complicated liver hydatid disease[13,18]. Surgery is the mainstay of treatment, although there is no consensus on the respective advantages of conservative and radical methods. Besides, in the treatment of peritoneal perforated cases, profuse peritoneal lavage with hypertonic solutions appears to be more necessary; in the setting of intrabiliary rupture, T-tube drainage, sphincteroplasty, and choledochoduodenostomy are appropriate treatment strategies to reduce the pressure in the biliary tree. In this study, we aimed to evaluate the predisposing factors for peritoneal perforation and intrabiliary rupture and the effects of these complications on morbidity, length of hospital stay (LOS), mortality, and recurrence in hydatid disease.

MATERIALS AND METHODS

Patients

We retrospectively evaluated medical records of 372 patients with liver hydatid cysts who were admitted to the Department of General Surgery, Faculty of Medicine, Erciyes University, between June 1990 and January 2008. In this period, 28 patients with peritoneal perforation (group Ⅰ), 93 patients with spontaneous intrabiliary perforation (group Ⅱ), and 251 patients with noncomplicated hydatid cysts (group Ⅲ) were treated in our clinics. The ratios of intrabiliary rupture and peritoneal perforation cases to noncomplicated cases were 37% and 11.1%, respectively.

Diagnosis

Patient age and sex, initial complaints, physical findings, cyst characteristics, imaging results, surgical procedures, reasons for peritoneal perforation and intrabiliary rupture, morbidity, LOS, recurrence rates, and mortality were evaluated. The patients with extrahepatic organ involvement were excluded.

Preoperative evaluation

The preoperative evaluation included blood tests (complete blood count, blood type, liver function tests), chest radiography, abdominal ultrasonography (US), and computed tomography (CT). Chest and abdominal radiography and abdominal US were performed in all patients at admission. The most important indications for CT were a need for additional anatomical and cystic details, the presence of multiple hydatid cysts, findings of a solid appearance, and findings of cyst infection. If intrabiliary rupture was suspected, magnetic resonance imaging (MRI) cholangiography was performed. Intraoperative ultrasound (IOUS) was used for identification of intraparenchimal cyst localization and selection of the best approach sight.

Surgical procedure

In all cases of liver hydatid cysts, the area around the cyst was covered with packs soaked in 3% hypertonic saline solution to prevent the further spread of the parasite during evacuation of the cyst. Cyst contents were aspirated from a suitable surface with a needle, which opened a nearly 1-2 cm incision; this was examined carefully for bile content. Subsequently, an aspirator was inserted into the cystic cavity to empty the remaining fluid, and then the incision was widened. The germinative membrane and daughter cysts were removed with forceps or spoons. With the roof excision of the redundant part of the cyst, an excellent exposure was obtained. After evacuation of the cyst, the cyst cavity was irrigated with 3% hypertonic saline for 10-15 min. Any orifices of bile ducts observed on the inner surface of the cavity were sutured with nonabsorbable sutures. To prevent peritoneal spreading in peritoneally perforated cases, the abdominal cavity was irrigated with 3% hypertonic saline or with povidone-iodine. Next, a surgical procedure, such as partial pericystectomy (PP) and capitonnage, PP and omentoplasty, PP and drainage, or PP and intraflexion, was performed. For pericystectomy, the hepatic pedicle was prepared for the Pringle maneuver. The cyst was then dissected along its boundary with healthy liver tissue. Blood vessels and small biliary structures passing through the plane between normal liver tissue and the cyst were clamped and divided. If the patient was fit and the cysts were away from the hepatic veins, large bile ducts, or major branches of the por-

tal vein and hepatic artery, pericystectomy was performed. Liver resections were performed only if multiple cysts were localized in one lobe and peripherally.

If the preoperative evaluation suggested a probability of a connection between the cyst and the biliary duct, or this was suspected during surgery, choledochotomy, placement of a T-tube, and an intraoperative cholangiographic and/or choledochoscopic evaluation were performed. In the case of any doubt about free biliary drainage, biliodigestive anastomosis or sphincteroplasty was added.

Medical treatment

Since 1993, medical treatment for hydatidosis has consisted of albendazole 10 mg/kg per day (Andazol, Biofarma, Istanbul, Turkey), beginning after surgery and continuing for 3 mo. Until 5 years ago, the therapy was performed over 3 mo and included 20 d of drug administration and 10 d drug free intervals. Over the last 5 years, the drug has been administered continuously with monthly blood liver function tests and complete blood count controls.

Follow-up

During follow-up, US was performed twice a year for 2 years, and then annually. CT scans were performed as needed.

Statistical analysis

Differences among categorical variables were compared using χ^2 tests, and Kruskal-Wallis tests were used for age, diameter, and number of cysts and LOS. Multinominal logistic regression analysis was performed to analyze the effects of variables (age; sex; previous hydatid disease surgery; location, number, position, and presence of cyst infection) influencing peritoneal perforation or biliary rupture. Data were analyzed with the SPSS software package (version 13.0; SPSS, Inc., Chicago, IL). Significance was set at $P < 0.05$.

RESULTS

The mean age (mean ± SD) of peritoneal perforated and intrabiliary ruptured patients was 36.32 ± 11.86 and 50.26 ± 13.88, respectively. When calculated, it was 45.62 ± 16.01 in the noncomplicated group. The mean age factor in the peritoneal perforation group was significantly different from others ($P = 0.001$). The sex ratio of the patients was not statistically different among the groups ($P = 0.95$, Table 1).

Previous hydatid disease surgery, cyst localization, number of cysts, and presence of infection were not statistically different among groups ($P > 0.5$; range, 0.109-0.683), whereas the differences in cyst diameters and positions (superficial or deep) were significant ($P = 0.001$ and 0.017, respectively; Table 1). Macroscopic appearance of an hydatid cyst that originates in the right lobe of the liver was seen in Figure 1.

The most common complaint was abdominal pain in all groups (67%). Other common complaints were nausea, vomiting, abdominal distension, and allergenic reactions in peritoneally perforated cases, jaundice (21%) in intrabiliary ruptured cases, and abdominal swelling (41%) in noncomplicated hydatid cysts. The most common physical examination findings were abdominal sensitivity and guarding (100%), acute abdomen findings (39%), distension (22%) in peritoneally perforated cases, hepatomegaly (28%) and jaundice (21%) in intrabiliary ruptured cysts, and finally, hepatomegaly (27%) in noncomplicated patients. In 49 (13%) patients, diagnosis was made incidentally by abdominal US or CT during a medical checkup or after a trauma.

When the predisposing factors for complications

Table 1 Patient and cyst characteristics of the three study groups

Characteristic	Group Ⅰ (n = 28)	Group Ⅱ (n = 93)	Group Ⅲ (n = 251)	P
Age[1] (yr)				0.001[b]
mean ± SD	36.32 ± 11.86	50.26 ± 13.88	45.62 ± 16.01	
Median (range)	37.0 (17-76)	53.0 (23-76)	43.0 (18-79)	
Sex				0.950
Male	15 (53)	51 (55)	141 (56)	
Female	13 (47)	42 (45)	110 (44)	
Previous hydatid disease surgery				0.204
No	24 (86)	74 (80)	219 (87)	
Yes	4 (14)	19 (20)	32 (13)	
Localization				0.109
Right lobe	17 (61)	54 (58)	178 (71)	
Left lobe	5 (18)	25 (26)	48 (19)	
Bilateral	6 (21)	14 (16)	25 (10)	
No. of cysts[1]				0.284
1	22 (78)	61 (66)	193 (77)	
2	4 (14)	17 (18)	39 (16)	
3	1 (4)	7 (8)	16 (6)	
4	1 (4)	5 (5)	3 (1)	
5	-	3 (3)	-	
Cyst diameter[1] (cm)	9.5 (4-24)	10 (6-29)	7 (3-31)	0.001[b]
Position				0.017[b]
Superficial	24 (86)	52 (56)	155 (62)	
Deep	4 (14)	41 (44)	96 (38)	
Cyst infection				0.683
Negative	26 (93)	89 (95)	234 (93)	
Positive	2 (7)	4 (5)	17 (7)	

Values are mean ± SD, median (range), or n (%). [b]Values indicate a significant difference. [1]Kruskal-Wallis test.

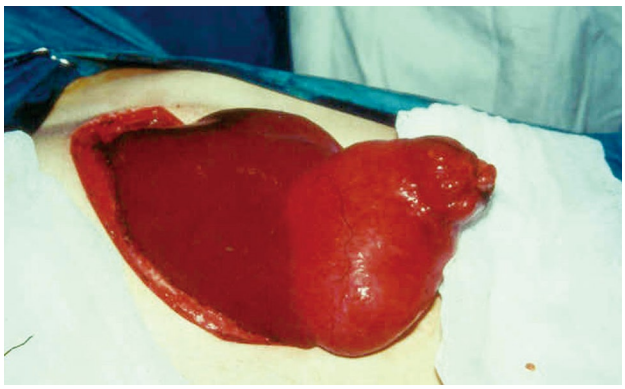


Figure 1 Macroscopic appearance of an hydatid cyst that originates in the right lobe of the liver.

Table 2 Multinominal logistic regression analyses of factors influencing peritoneal and biliary perforation of liver hydatid cysts

Factor	Group Ⅰ *vs* group Ⅲ		Group Ⅱ *vs* group Ⅲ	
	Odds ratio	95% CI	Odds ratio	95% CI
Sex				
Male	1.00		1.00	
Female	1.61	0.68-3.77	1.10	0.65-1.87
Age	0.59	0.41-0.84	1.30	1.07-1.57
Occurrence type				
Primary occurrence	1.00		1.00	
Recurrence	0.81	0.23-2.77	1.33	0.66-2.65
Localization				
Unilobar	1.00		1.00	
Bilateral	1.48	0.86-2.55	1.54	1.07-2.21
No. of cysts	1.21	0.65-2.23	1.80	1.30-2.50
Cyst diameter (cm)	1.11	1.04-1.19	1.12	1.07-1.57
Position				
Superficial	1.00		1.00	
Deep	0.27	0.09-0.84	1.40	0.18-4.33
Cyst infection				
Positive	1.00		1.00	
Negative	1.02	0.20-5.11	0.58	0.17-2.01

Group Ⅰ = 28, group Ⅱ = 93, group Ⅲ = 251 (reference value).

were evaluated, younger age, superficial position, and larger cyst dimensions increased peritoneal perforation rates. It has been seen that older age, increased cyst dimensions, and presence of multiple and bilobar cysts increases intrabiliary rupture rates. Sex, primary or recurrent disease, and cyst infection were not as effective variables in both logistic models (Table 2).

Abdominal US was done in 326 (87.6%) patients and demonstrated hepatic cysts with 96.7% sensitivity (Figure 2). CT was used as the second most frequent (n = 148, 46%) diagnostic modality, with 96.5% sensitivity. In 24 (6.5%) patients with intraparenchymal cysts, IOUS was performed for identification of cyst localization and selection of the best approach sight. MRI cholangiography was performed after 1996 to assess the spread of disease to the biliary tree but was not used routinely and was done in 37 patients (9.9%) (Figure 3). The indications for MRI cholangiography are the presence of jaundice seen during the physical examination, elevation of serum bilirubin and alkaline phosphatase levels, and other obstructive jaundice symptoms and demonstrated 98% sensitivity.

Surgical procedures used for the cysts of the 372 patients are shown in Table 3. Partial pericystectomy and drainage was the most frequent surgical procedure in all groups. Three of 4 jaundiced patients underwent cholecystectomy plus T-tube drainage, and the other patient underwent cholecystectomy plus sphincteroplasty in the peritoneal perforation group. In all intrabiliary rupture cases, cholecystectomy and common bile duct exploration were performed. Intraoperative cholangiography was performed in all patients, and choledochoscopy was also performed in 62 (66.6%) patients. Cyst remnants and daughter vesicles in the bile ducts were evacuated

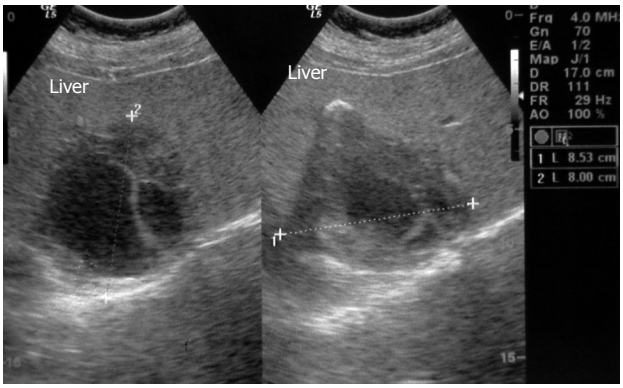


Figure 2 Ultrasonographic appearance of an hydatid cyst of the liver.

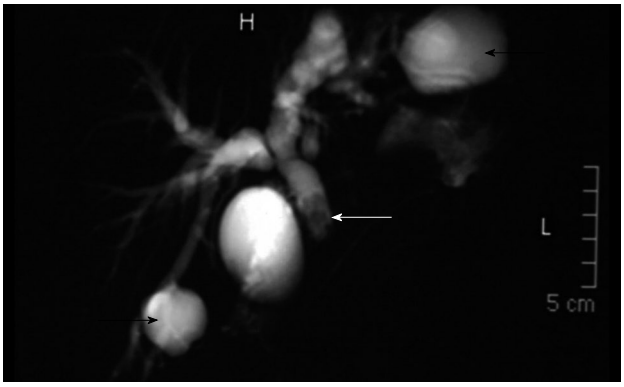


Figure 3 Magnetic resonance imaging cholangiography. Two biliary ruptured (right and left lobe) hydatid cysts (black arrows) and daughter vesicles in common bile duct (white arrow).

with Dormia forceps or a Fogarty catheter in 16 (17.2%) patients. A T-tube drainage was performed in 72 (77.4%) patients, choledochoduodenostomy was performed in 15 (16.1%) patients, and sphincteroplasty was performed in 6 (6.4%) patients.

The most frequent postoperative complications were wound infection (5.6%) and pulmonary complications (2.9%). In the peritoneal perforation group, 10 surgical complications occurred in 7 (25%) patients; in the intrabiliary rupture group, 18 complications occurred in 15 (16.1%) patients; and in the noncomplicated group, 16 complications occurred in 14 (5.5%) patients. When compared, complication rates were significantly different (P = 0.002, Table 4). When compared according to Dindo & Clavien Classification of Surgical Complications[19], the differences between grade Ⅰ, grade Ⅳa and grade Ⅳb surgical complications were not significant (P > 0.05, range, 0.08-0.57), however differences between grade Ⅱ, grade Ⅲa and grade Ⅲb complications were statistically significant (P < 0.05; range, 0.003-0.036).

Median postoperative LOS in the peritoneal perforation group, intrabiliary ruptured group, and noncomplicated group were 12 (range, 5-21), 13 (range, 5-37), and 9 (range, 4-19) d, respectively. When LOS was compared, there was no significant difference between the groups (P > 0.05). According to the additional surgical procedures,

Table 3 Surgical procedures used

Surgical procedure	No. of cysts (%)		
	Group Ⅰ (*n* = 28)	Group Ⅱ (*n* = 93)	Group Ⅲ (*n* = 251)
Main surgical procedure			
PP and drainage	26 (70.3)	101 (67.8)	231 (73.5)
PP and omentoplasty	2 (5.4)	13 (8.7)	26 (8.3)
PP and capitonnage	2 (5.4)	11 (7.4)	20 (6.4)
PP and intraflexion	-	3 (2.0)	8 (2.6)
Pericystectomy	5 (13.5)	14 (9.4)	19 (6.0)
Liver resection	2 (5.4)	7 (4.7)	10 (3.2)
Total No. of cysts	37	149	314
Additional surgical procedure			
Choledochotomy and T-tube	3 (10.7)	77 (82.8)	7 (2.7)
Choledochotomy and choledocho-duodenostomy or sphincteroplasty	1 (3.5)	16 (17.2)	2 (0.8)

PP: Partial pericystectomy.

Table 4 Morbidity, recurrence, and mortality rates of the three study groups *n* (%)

	Group Ⅰ (*n* = 28)	Group Ⅱ (*n* = 93)	Group Ⅲ (*n* = 251)	*P*
Medical complications[1]				0.163
Cardiac	1 (3.6)	1 (1.1)	3 (1.2)	
Respiratory	2 (7.2)	3 (3.2)	6 (2.4)	
Other	1 (3.6)	2 (2.2)	4 (1.6)	
Surgical complications				
Wound infection	6 (21.4)	6 (6.5)	9 (3.5)	0.001[b]
Biliary fistula	1 (3.6)	9 (9.7)	2 (0.8)	0.001[b]
Intra-abdominal abscess	-	1 (1.1)	2 (0.8)	0.855
Incisional hernia	2 (7.2)	2 (2.2)	3 (1.2)	0.087
Gastrocutaneous fistula	1 (3.6)	-	-	0.002[b]
Recurrence	3 (10.7)	3 (3.2)	8 (3.2)	0.132
Mortality	-	1 (1.1)	3 (1.2)	0.844

[b]Values indicate a significant difference. [1]Cardiac complications included heart failure and myocardial infarction; respiratory complications included atelectasis, pneumonia, adult respiratory distress syndrome, and pulmonary embolus; other complications included urinary tract infection and acute thromboembolic disease of the lower extremities.

the median LOS was 8 (range, 4-19) d in patients on which choledochotomy had not been performed, compared to 15 (range, 11-37) d in the choledochotomy with T-tube group and 10 (range, 6-13) d in the bilioenteric anastomosis group. There was a significant difference in LOS between the groups with choledochotomy with T-tube and without choledochotomy ($P < 0.05$). In contrast, no difference between the groups with biliodigestive anastomosis and without choledochotomy was seen ($P > 0.05$).

The follow-up data were complete for 28 (100%) of the peritoneal perforated group, 76 (82%) of the intrabiliary ruptured group, and 198 (79%) of the noncomplicated group. The mean follow-up periods were 37.3 ± 26.3 (range, 2-144) mo. The overall recurrence rate was 3.8% (14 patients), but there was not any statistical difference among the patient groups ($P = 0.13$, Table 4). The early postoperative mortality rate was 1.1% ($n = 4$). The causes of death were pulmonary embolism in one patient, myocardial infarct in one patient, and postoperative hemorrhage in two patients (Table 4). These causes were identified clinically, without necropsy.

DISCUSSION

Peritoneal perforation into the abdominal cavity and the spontaneous intrabiliary rupture of liver hydatid disease are not rare complications and cause serious problems. In our study, the ratios of peritoneal perforation and intrabiliary rupture cases to noncomplicated cases were 11.1% and 37%, respectively. The most common complication is rupture of the cyst, either internally or externally, followed by anaphylactic reaction and jaundice[5,9,10]. Systemic anaphylactic reactions have been reported in 1% to 12.5% of patients with intraperitoneal perforation, and these reactions may be life threatening[9,10]. Jaundice is the most important sign of intrabiliary rupture within a range of 9% to 30%[20,21]. In patients with peritoneal perforation and intrabiliary rupture, specific management has not been evaluated sufficiently. The presence of complications must be considered in the surgical treatment.

One third of 372 patients (32.5%) who underwent an operation for liver hydatid disease presented with complications. When compared, sex factor was not statistically different among groups ($P = 0.95$), but the mean age in the peritoneal perforated group was significantly different ($P = 0.001$, Table 1). Trauma is the most frequent etiological cause of perforation[9,22]. No patients in the current series experienced spontaneous peritoneal rupture. Hydatidosis is endemic in Turkey and the frequency of traffic accidents there is high[23]. Traffic accidents generally affect younger individuals, and the age risk factor found in our study may relate to this phenomenon. In superficial and bigger cysts, lack of normal liver tissue around the cyst to protect against trauma may be a cause of frequent rupture.

The differences in factors such as previous hydatid disease surgery, cyst localization, cyst number, and cyst infection were not significant among groups ($P > 0.05$, range, 0.109-0.683). Besides age, cyst diameter and cyst position (superficial or deep) were also significantly different factors ($P = 0.001$ and 0.017, respectively; Table 1). When compared to the same parameters with multinominal logistic regression analyses, young age, superficial position, and large diameter are significant predisposing factors for peritoneal perforation (Table 2). The hydatid cyst wall consists of an inner germinal layer and an outer laminated membrane. Surrounding the wall is the pericyst, which has an outer zone of compressed liver cells. A lack of presence of normal liver tissue around superficial cysts plays a role in relatively higher perforation rates. Similarly, an increase in cyst diameter may increase perforation risk either by becoming more superficial during growth or by increasing intracystic pressure.

Additionally, liver hydatid cysts at the dome of the liver grow progressively and have a tendency to erode

the diaphragm[24]. In our study, it has been shown that older age, bigger dimensions, multiplicity of the cysts, and bilobar cysts are predisposing factors for intrabiliary rupture. The relationship between older age and intrabiliary rupture probably depends on cyst age. Hydatid disease is a slowly growing disease, and in early times, most of the cysts produce no symptoms. In advanced cases, the intracystic pressure may cause tension on cyst walls and thinner wall formations. The communications between the cysts and the biliary tree usually involve small cholangioles, and direct extension into major bile ducts is rare[25,26]. The most probable theory may be the fenestration of the cyst and minor bile ducts, which have low pressure in the surrounding liver tissue. In the case of multiple and bilobar cysts, this communication rate may increase. Factors such as sex, primary or recurrent disease, or infection did not show effectiveness in both logistic models (Table 2).

Cyst dimension and age (younger age for peritoneal perforation and older age for intrabiliary rupture) have been determined as significant predisposing factors for the two complications. We do not believe a direct relationship exists between the two complications, but we can speculate that risk of peritoneal perforation risk may decrease in intrabiliary ruptured cysts because of the lower intracystic pressure probability. In our study the ratio of intrabiliary ruptured cysts was 97 out of 372 (26%), whereas intrabiliary rupture was 4 out of 28 (14.3%) peritoneally perforated cases, but this was not statistically significant.

The principles of hydatid surgery are (1) removal of all infective cyst parts; (2) inactivation of the cyst cavity; (3) avoidance of intra-abdominal spillage of cyst contents; and (4) management of the residual cavity. A lot of surgical techniques have been proposed for liver hydatid disease during the last three decades. Some authors suggest that obliteration procedures, such as capitonnage, intraflexion, or omentoplasty, are simple and safe methods[27]. In the past, obliteration procedures such as omentoplasty, capitonnage, and intraflexion were used for most patients in our clinic. However, in the last 10 years use of these management options decreased gradually and for the last 5 years we have not shown a preference for them. Recently, some authors have favoured the use of pericystectomy and liver resections because complete surgical resection is the ideal treatment for hydatid disease[18]. In our study, liver resections were performed only if multiple cysts were localized in one lobe, located peripherally, and were pedunculated, and pericystectomy was performed if the cysts were away from the major vascular and biliary structures of the liver. The most important advantages of these resections are that no dead space is left behind and that all the cyst wall is removed, which prevents liver regeneration. The need for sufficient technical infrastructure and surgical experience in the field of hepatic surgery limit these treatments in small centers, especially in developing countries. Relatively small-sized subcapsular cysts can be managed by nonanatomic resection of the cysts with a rim of healthy hepatic tissue but routine application of pericystectomy and liver resection may increase the operative complications, such as bleeding and postoperative morbidity and mortality.

For almost 15 years, our preferred surgical technique for treatment of liver hydatid cysts has been PP and drainage, which has been performed in 70% of the patients (Table 3). Leaving the cyst cavity open, the peritoneum has more support in recent years[13]. After controlling the relation between the biliary tree, it has been stated that this procedure does not increase recurrence or cause adhesion. The most important superior aspect of this technique is its applicability to every cyst at every localization. Other advantages of the procedure are the simplicity, no requirement of advanced experience, effectiveness, and a shorter operation time. The most important detail of this procedure in our experience is to perform the partial pericystectomy as widely as possible, and the drainage procedure should be added after the cyst contents are removed completely. After the cyst management, in peritoneal perforated cysts, the most important step is irrigation of the peritoneal cavity with a sufficient amount of scolicidal agents and removal of all cystic content, and in intrabiliary ruptured cases, clearance of the cystic material from the biliary tree is most important; also, the restoration of normal bile flow should be performed. The fistula was large enough to allow daughter cysts and debris to migrate into the biliary tree. For this reason introperative cholangiography was performed in all patients who presented with abnormal liver function tests preoperatively, bile-stained cyst fluid, and bile leak from the cyst wall. During the introperative cholangiography, visible bile fistulas must be sutured by using late absorbable suture materials.

After the intervention to the cyst, the most important step is irrigation of the cyst cavity with a sufficient amount of scolicidal agents and careful removal of all cystic content from the patient. Numerous solutions, such as hypertonic saline solutions (15%-30%), have been used as scolicidal agents for the purpose of inactivation[5,28]. The concentration of hypertonic saline solutions has diminished gradually. Now we use only 3% concentrations. Gargouri *et al*[29] used 3%-5% saline and stated that the application time is more important than the concentration. Although Derici *et al*[30] reported that hypertonic saline is not appropriate because it may damage the peritoneal surfaces and may cause hypernatremia, we have not encountered any significant complications with the use of this solution. Additionally, we believe that profuse peritoneal lavage with hypertonic sodium chloride is mandatory for preventing intra-abdominal recurrence of intraperitoneal ruptured hydatid disease.

The most effective surgical procedures following the cleaning of hydatid material from the biliary tree remain controversial. Some authors prefer T-tube[13-15]; in contrast, some reported that wide choledochoduodenostomy decreases morbidity and mortality[31]. In our study, complication rates in patients with bilioenteric anastomosis and T-tube were 21% and 27%, respectively.

In the literature, reported complication rates are be-

tween 6% and 47%; recurrence rates are 8% to 15%[32]. In this study, the overall postoperative complication and recurrence rates were 18% and 3.8%, respectively. Most often, surgical complications were wound infection (n = 21) and biliary fistulas (n = 12); the difference between groups was significant (P = 0.001, Table 4). The important causes of higher wound infection rates in the peritoneal perforation group were coinjuries, which were seen in 10 patients. There were small intestine and colon perforations in five cases; in addition, splenic, pancreatic, and renal injuries were each seen in one patient. Retroperitoneal hematoma was seen in the remaining two coinjured patients. External biliary fistulas did not develop in 68 of the T-tube patients and in none of the biliodigestive anastomosis or sphincteroplasty patients in the intrabiliary rupture group. T-tubes were removed after a control cholangiography, which was done after a mean period of 12 d postoperatively. External biliary fistulas developed in 9 (9.7%) patients. If the fistulas did not close with conservative follow-up, endoscopic sphincterotomy was performed after a mean period of 19 d. The causes of fistulas were remnant germinal vesicles and debris in the distal common bile duct in 5 patients and papillary stenosis in 4. In the peritoneally perforated and noncomplicated groups, biliary fistulas developed in 3 patients who had hepatic resections, and the problem was resolved with endoscopic sphincterotomy. Endoscopic procedures have been used not only for diagnostic purposes, but for treatment as well. Dumas *et al*[33] treated 7 of 28 patients with cholangitis due to cystic material with ERCP. Tekant *et al*[34] and Vignote *et al*[35] reported that endoscopic sphincterotomy achieved closure of fistulas within 5-7 d. In our series, fistulas closed in all external biliary fistula patients within a mean time of 7 (range, 3-17) d. At our center, intraoperative cholangiography with or without subsequent duct exploration remains the method of choice to prevent or decrease incidence of associated injuries and external biliary fistula. Several authors have advocated pre-operative or post-operative ERCP[33-35]. If performed in conjunction with a sphincterotomy, an ERCP may be used to clear the biliary tree as a planned procedure, thereby avoiding the need for an intraoperative cholangiography and bile duct exploration.

Recurrence occurred in 14 nonperforation cases (3.8%). When compared with the reported rates, the recurrence of our study is low. The cause of this may be the routine use of albendazol for 3 mo postoperatively. Recurrence rates were not significantly higher in peritoneal perforation and intrabiliary rupture cases when compared with noncomplicated cases (P = 0.132, Table 4). Recurrences of hydatid disease are usually due to inadequate cystic content removal, spillage of cystic liquid intraoperatively, undetected cysts, and satellite lesions and reinfestations. Readmissions in our series due to recurrent disease were generally after a long time and mostly from rural areas. Another clinical finding that suggests reinfestation is the presence of hydatid cyst at the same localisation which seen was 4 of 14 recurrent cases. For these reasons, the probability of reinfestation is high in endemic countries like Turkey. When compared with the literature, in our study the recurrence rate was low. Albendazole treatment may play an important role in this low rate. Although the efficacy of perioperative prophylaxis with albendazole has been controversial, recent studies suggest that medical treatment is effective in the prevention of recurrence after surgery[36,37]. To diminish recurrence and potential spread of the organism, we believe that medical treatment should apply as an adjuvant therapy to surgery when complete removal of the cyst is impossible or when the cyst contents threaten to disseminate due to peritoneal rupture. It is indicated in patients who are at high risk for surgery, and in the presence of bilateral multiple small cysts. Those with small cysts (< 4 cm) and cysts with thin walls are the best group for effective medical treatment. In addition, exogenous vesiculations have been pronounced in hydatid cyst recurrences[38]. The importance of medical treatment which is mainly effective on small cysts, may indicate the usefulness of the effect on recurrence.

Although there is insufficient published evidence to support a clear recommendation for the use of albendazole preoperatively[39], we highly recommend this treatment for one week before surgical exploration. Preoperative therapy may reduce the risk of intraperitoneal seeding of infection that develops *via* cyst rupture and spillage occurring spontaneously or during surgery. In our study no correlation was found between recurrence of cysts and albendazole use.

Serology detect specific serum antibodies or circulating antigen by a variety of immunodiagnostic methods. Of these, the enzyme immunosorbent assay (ELISA) employing hydatid fluid antigen for detection of echinococcal antibodies (IgG) in the serum is the most widely used. Serological tests are of little clinical use in monitoring patients after operation for cystic echinococcosis or patients on chemotherapy. The antibody titers rise following surgical treatment. The titers start falling at 3 mo and become negative in a period of 12-24 mo. False positive tests can occur in normal persons from endemic areas and in those with other parasitic infections[16]. In our study, 86 (58.9%) of 146 (42.4%) serologically evaluated patients of group Ⅱ and Ⅲ were positive, and 41 (28%) of these were also serologically positive 2 years after surgery. However, recurrence occurred in only 1 of the patients that was serologically positive for a long term. We think that the value of serological screening in areas endemic for cystic echinococcosis is enhanced by combining it with abdominal US.

Mortality rates of the study groups were not statistically different (P = 0.814, Table 4). Liver resections were performed in two mortality cases that were due to intraoperative and postoperative bleeding. One of the patients who had PP and drainage died because of myocardial infarct, and one patient had PP and omentoplasty cases of pulmonary thromboembolism. When compared, mortality rates were significantly different among groups (P < 0.001). This result requires discussion of the controversial radical methods in hydatid disease. Chautems *et al*[38] recommended complete resection of hepatic hydatid cysts. In contrast, Daali *et al*[40] preferred

conservative procedures. We think that radical methods should not be performed routinely for such a slowly growing benign pathology. It is wise to reserve radical surgery for peripheral, easily accessible cysts and for unilober multiple cysts.

LOS did not differ between the cases of peritoneal perforation and intrabiliary rupture when compared with noncomplicated cases ($P > 0.05$), whereas, according to the additional surgical procedures, there was a significant difference in LOS between choledochotomy with T-tube and procedures without choledochotomy ($P < 0.05$). An external biliary fistula formation and T-tube procedure, wherein the T-tube may be taken out after approximately 7-10 d, may affect LOS. In contrast, there was no difference between the groups with biliodigestive anastomosis and without choledochotomy ($P > 0.05$). Biliodigestive anastomosis may be a good alternative surgical procedure to T-tubes, with lower complication rates and shorter LOS.

According to this study's multinominal logistic regression analysis, peritoneal perforation risk increases with younger age, superficial localization, and larger dimensions of cysts, and older age, multiple and larger cysts, and bilobar localization are established as predisposing factors for intrabiliary rupture. Morbidity rates significantly increased in peritoneally perforated and intrabiliary ruptured cases, but there was no difference in recurrence rates and hospital stay times. Our preferred surgical technique for liver hydatid cysts is partial pericystectomy and drainage because the rate of postoperative complications and recurrence, and the duration of hospital stay are satisfactory. We believe that radical methods should not be performed routinely for such a benign pathology. The localization and number of cysts, relation to blood vessels and bile ducts, and general status of the patient are parameters influencing the choice of surgical procedure. In peritoneal perforated and intrabiliary ruptured cysts, the most important steps are irrigation of the peritoneal cavity with a sufficient amount of scolicidal agents, removal of all cystic contents from the peritoneum, and clearance of the cystic material from the biliary tree. However, randomized controlled studies are needed to establish the best surgical management for peritoneal perforated and intrabiliary ruptured liver hydatid cysts.

COMMENTS

Background

The development of hydatid disease associated with *Echinococcus granulosus* is still an important health problem. The clinical picture can be severe, and complications may occur, making the already difficult treatment even more so. Complications are observed in one third of patients with liver hydatid cysts. The most common complication is rupture of the cyst, either internally or externally.

Research frontiers

Only a few studies have reported the management and outcome of patients with complicated liver hydatid disease. Surgery is the mainstay of treatment, although there is no consensus on the respective advantages of conservative and radical methods. In this study, the authors evaluated the predisposing factors for peritoneal perforation and intrabiliary rupture and the effects of these complications on morbidity, length of hospital stay, mortality, and recurrence in hydatid disease.

Innovations and breakthroughs

According to this study's multinominal logistic regression analysis, peritoneal perforation risk increases with younger age, superficial localization, and larger dimensions of cysts, and older age, multiple and larger cysts, and bilobar localization are established as predisposing factors for intrabiliary rupture. Morbidity rates significantly increased in peritoneally perforated and intrabiliary ruptured cases, but there was no difference in recurrence rates and hospital stay times.

Applications

In peritoneal perforated and intrabiliary ruptured cysts, the most important steps are irrigation of the peritoneal cavity with a sufficient amount of scolicidal agents, removal of all cystic contents from the peritoneum, and clearance of the cystic material from the biliary tree. However, randomized controlled studies are needed to establish the best surgical management for peritoneal perforated and intrabiliary ruptured liver hydatid cysts.

Terminology

Echinococcus granulosus is a parasitic tapeworm that lives in the bowels of dogs. Humans contract the disease through enteral exposure and become accidental intermediate hosts.

Peer review

Alper Akcan *et al*, evaluate the predisposing factors for peritoneal perforation and intrabiliary rupture and the effects of these complications on surgical outcome in liver hydatid disease. This is one of the largest and well treated series of patients that had been publish. The structure and methodology followed by the authors is correct in my opinion and the techniques they applied for the treatment of these complex patients are modern and correct.

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BRIEF ARTICLE

Is inconsistency of α-fetoprotein level a good prognosticator for hepatocellular carcinoma recurrence?

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Abstract

AIM: To identify the clinical outcomes of hepatocellular carcinoma (HCC) patients with inconsistent α-fetoprotein (AFP) levels which were initially high and then low at recurrence.

METHODS: We retrospectively included 178 patients who underwent liver resection with high preoperative AFP levels (≥ 200 ng/dL). Sixty-nine HCC patients had recurrence during follow-up and were grouped by their AFP levels at recurrence: group Ⅰ, AFP ≤ 20 ng/dL (n = 16); group Ⅱ, AFP 20-200 ng/dL (n = 24); and group Ⅲ, AFP ≥ 200 ng/dL (n = 29). Their preoperative clinical characteristics, accumulated recurrence rate, and recurrence-to-death survival rate were compared. Three patients, one in each group, underwent liver resection twice for primary and recurrent HCC. AFP immunohistochemistry of primary and recurrent HCC specimens were examined.

RESULTS: In this study, 23% of patients demonstrated normal AFP levels at HCC recurrence. The AFP levels in these patients were initially high. There were no significant differences in clinical characteristics between the three groups except for the mean recurrence interval (21.8 ± 14.6, 12.3 ± 7.7, 8.3 ± 6.6 mo, respectively, P < 0.001) and survival time (40.2 ± 19.9, 36.1 ± 22.4, 21.9 ± 22.0 mo, respectively, P = 0.013). Tumor size > 5 cm, total bilirubin > 1.2 mg/dL, vessel invasion, Child classification B, group Ⅲ, and recurrence interval < 12 mo, were risk factors for survival rate. Cox regression analysis was performed and vessel invasion, group Ⅲ, and recurrence interval were independent risk factors. The recurrence interval was significant longer in group Ⅰ (P < 0.001). The recurrence-to-death survival rate was significantly better in group Ⅱ (P = 0.016). AFP staining was strong in the primary HCC specimens and was reduced at recurrence in group Ⅰ specimens.

CONCLUSION: Patients in group Ⅰ with inconsistent AFP levels had a longer recurrence interval and worse recurrence-to-death survival rate than those in group Ⅱ. This clinical presentation may be caused by a delay in the detection of HCC recurrence.

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Key words: Hepatocellular carcinoma; Inconsistent α-fetoprotein; Outcome; Recurrence

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INTRODUCTION

Serum α-fetoprotein (AFP) level is widely used as a tumor marker for the diagnosis and detection of both occurrence and recurrence of hepatocellular carcinoma (HCC)[1,2]. For a patient with a history of liver disease, consensus for the diagnosis of HCC can be achieved with an elevated AFP level (≥ 200 ng/dL), along with a dynamic imaging study showing a hepatic tumor, with enhancement in the arterial phase and wash-out in the venous phase[3,4].

A high serum AFP level in HCC also indicates poor tumor differentiation, larger tumor burden, recurrence after tumor resection, and an unfavorable outcome[5-10]. In clinical practice, a dramatic decline in serum AFP level indicates that the tumor has been surgically removed[11]. The AFP level is broadly applicable both pre- and postoperatively.

AFP is secreted in only about 70% of HCC cases, so both false-negative and false-positive rates are high when using AFP as the serological marker for the detection of HCC[12]. In general, patients have an initially high serum AFP level when HCC is diagnosed. Serum AFP level is also considered an effective tumor marker in the surveillance of HCC recurrence[13,14]. Recurrent HCC is expected to have the same bioactivity as the primary tumor and to secrete high levels of AFP, making AFP a seemingly reliable choice. Regular checks of serum AFP levels in patients who initially had high AFP levels to identify HCC recurrence is suggested as a practice guideline by the National Comprehensive Cancer Network (NCCN) version 1, 2009[15].

However, inconsistent AFP levels have been found in patients diagnosed with HCC where initial AFP levels were high, but were normal when HCC recurred. A true relapse of HCC and a second primary tumor due to chromosomal aberration are possible mechanisms[16], and inconsistent AFP levels may differentiate them *via* AFP secretion bioactivity. If we depend on the AFP level to signal an HCC recurrence, we may miss or delay the diagnosis of recurrence. We may expect the clinical outcome of patients with inconsistent AFP levels to be similar to those with normal AFP levels. However, this clinical presentation has not been investigated before. Therefore, it is important to study the prognosis of this subgroup of HCC patients whose AFP levels are high initially and normal at recurrence. It is possible that the findings of this study could modify the surveillance principle for this subgroup of HCC patients and improve the quality of medical care.

MATERIALS AND METHODS

Patients

From 1996 to 2008, 642 patients newly diagnosed with HCC who underwent liver resection were entered into a computer database at the Tri-Service General Hospital, a tertiary referral medical center in Taipei, Taiwan. The diagnosis of HCC in all patients was established histologically from surgical specimens. HCC patients with an initial serum AFP level ≥ 200 ng/dL (n = 178) were selected and 82 had HCC recurrence during follow-up. Thirteen patients were excluded because of an American Joint Committee on Cancer (AJCC) stage Ⅳ diagnosis, lost to follow-up or missing data. Therefore, 69 patients were included in this study and grouped according to their serum AFP levels when HCC recurred (Figure 1).

Grouping by AFP level at recurrence

Sixteen patients with normal AFP levels (≤ 20 ng/dL) during HCC recurrence were assigned to group Ⅰ; 24 patients with AFP levels between 20 ng/dL and 200 ng/dL were assigned to group Ⅱ; and, 29 patients with AFP levels of 200 ng/dL or higher were assigned to group Ⅲ. In our hospital, the normal AFP level is ≤ 20 ng/dL measured by radioimmunoassay using an anti-AFP antibody (ELSA 2-AFP kit, CIS Bio International, Cedex, France).

Comparing the parameters of each group

Preoperative clinical characteristics were collected from the database and compared. These characteristics included age, gender, tumor size, serum alanine aminotransferase (ALT) level, total serum bilirubin, serum albumin level, platelet count, vessel invasion, tumor number, clinical stage (AJCC, Child-Pugh classification), hepatitis B virus, hepatitis C virus, presence of cirrhosis, mean recurrence interval, mean recurrence-to-death time, mean survival time and location of recurrence. Salvage treatments for recurrent HCC included repeat liver resection, liver transplantation, transarterial chemo-embolization, radiofrequency ablation, and thalidomide therapy. The mean AFP levels at operation (AFP op) and at recurrence (AFP recur) were calculated for these three groups. Intra-hepatic metastasis was defined as recurrent HCC located at the adjacent segment, section and same lobe. In contrast to intra-hepatic metastasis, multicentric recurrence was defined as recurrent HCC located at the contralateral liver lobe. The pathological grade was defined as: grade Ⅰ - well differentiated, grade Ⅱ - moderate, and grade Ⅲ - poorly differentiated, according to the report by the pathologist (Dr. Yu CP).

Endpoint

The mean recurrence interval was defined as the interval between the operation and HCC recurrence. The starting point of overall survival was the operation date, and recurrence-to-death was the date of recurrence diagnosis. The endpoint of accumulated recurrence rate was defined as the date of recurrence and the endpoint of recurrence-

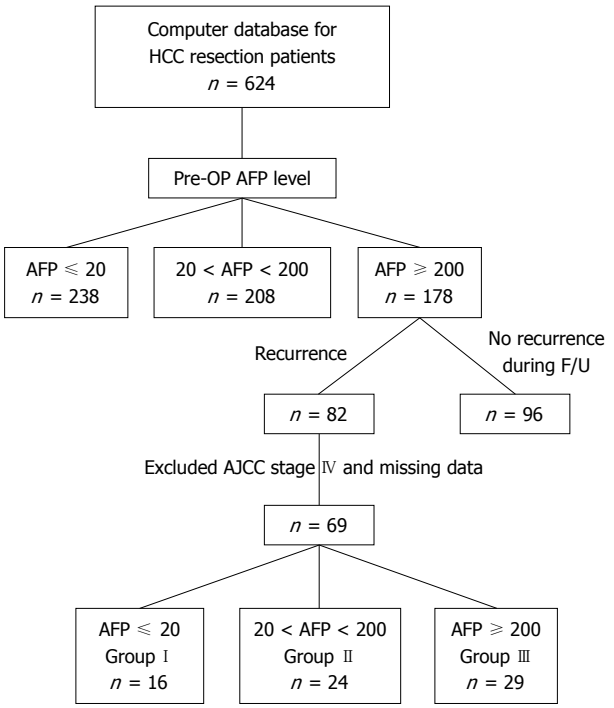


Figure 1 Flow chart for patient selection. HCC: Hepatocellular carcinoma; AFP: α-fetoprotein; AJCC: American Joint Committee on Cancer.

to-death survival was the date of death or the last follow-up date if the patients survived. The choice of salvage therapy was according to the willingness of the patient, the degree of tumor progression, and the criteria for treatment guidelines of the Cancer Committee at the Tri-Service General Hospital. The recurrence interval was defined as the day of the initial liver resection to the day of HCC recurrence diagnosis.

AFP immunohistochemistry

Three patients, one from each group, underwent a second liver resection for tumor recurrence. Surgical specimens from both operations in each of these three patients were reviewed by a pathologist (Dr. Yu CP). The specimens were examined by hematoxylin and eosin staining and immunohistochemistry for AFP levels and the differences were compared.

Follow-up

All patients were followed up regularly every month in the first year and every 3 mo thereafter. AFP level was monitored at each follow-up. Abdominal ultrasound imaging or computed tomography (CT) was performed every 3-6 mo in first two years, then annually, according to the NCCN practice guideline[15]. In some patients, magnetic resonance imaging, hepatic angiography, and percutaneous needle biopsy were considered if the CT images were not conclusive for a new hepatic lesion. All clinicopathological data were collected in the computer databases of the Cancer Board at the Tri-Service General Hospital, and the follow-up data were regularly updated. This study was approved by the Institutional Review Board of Tri-Service General Hospital. The follow-up period of this study ended on June 30, 2009.

Table 1 Clinical characteristics of groups Ⅰ, Ⅱ, and Ⅲ

	Group Ⅰ (*n* = 16)	Group Ⅱ (*n* = 24)	Group Ⅲ (*n* = 29)	*P*
Age (yr)	59.7 ± 12.5	57.1 ± 16.6	48.8 ± 16.3	0.055
Gender (M/F)	10/6	12/12	19/10	0.498
Tumor size (cm)	5.64 ± 3.07	4.80 ± 3.40	5.08 ± 3.11	0.696
ALT (U/L)	74.1 ± 50.9	77.2 ± 84.4	60.6 ± 44.1	0.615
T Bil (mg/dL)	0.79 ± 0.32	0.88 ± 0.49	0.96 ± 0.54	0.496
Albumin (g/dL)	3.74 ± 0.60	3.99 ± 0.50	3.74 ± 0.55	0.196
Plat (10^3/μL)	154.0 ± 65.8	134.9 ± 68.3	181.1 ± 94.5	0.155
Vessel invasion (+/-)	4/12	6/18	8/21	0.971
Tumor number (S/M)	9/7	16/8	15/14	0.541
AJCC Ⅰ/Ⅱ/Ⅲ	6/2/8	14/4/6	9/6/14	0.282
Child A/B	13/3	22/2	23/6	0.445
HBV (+/-)	12/4	15/9	25/4	0.241
HCV (+/-)	5/11	7/14	6/23	0.678
Cirrhosis (+/-)	10/6	14/10	20/9	0.720
AFP (op) (ng/dL)	1582 ± 2645	3049 ± 7215	2776 ± 6696	0.751
AFP (recur) (ng/dL)	7.5 ± 5.2	86.8 ± 57.9	8162.3 ± 15250.7	0.006
Recur interval (mo)	21.8 ± 14.6	12.3 ± 7.7	8.3 ± 6.6	< 0.001
Recur-to-death (mo)	18.4 ± 19.5	24.1 ± 19.8	13.6 ± 19.6	0.162
Survival time (mo)	40.2 ± 19.9	36.1 ± 22.4	21.9 ± 22.0	0.013
Intra-hepatic recur	2/14	8/16	17/8	0.001

ALT: Alanine aminotransferase; T Bil: Total bilirubin; Plat: Platelet; Child: Child-Pugh classification; AJCC: American Joint Committee on Cancer; HBV (+): Hepatitis B virus infection; HCV (+): Hepatitis C virus infection; Cirrhosis (+): Cirrhotic change by pathologic finding; AFP (op): α-fetoprotein level preoperatively; AFP (recur): α-fetoprotein level at tumor recurrence; Recur interval: The interval from operation to tumor recurrence; Recur-to-death: The interval from tumor recurrence to patient's death; Survival time: The time from operation to last follow-up date or death date; Intra-hepatic recur: Hepatocellular carcinoma (HCC) recurrence located at the adjacent segment, section and same lobe/HCC recurrence located at contralateral liver lobe.

Statistical analysis

Continuous clinical data were analyzed using the Student's *t* test and ANOVA, and the categorical data were analyzed using Fisher's exact test or the χ^2 test. Kaplan-Meier and Cox regression analyses were performed to determine the survival curves, and the survival relative ratio between the three groups of HCC patients. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using statistical software (SPSS version 15.0 for Windows, Chicago, IL, USA).

RESULTS

Of the 69 HCC patients with initially high AFP levels who underwent liver resection, 16 had normal serum AFP levels (⩽ 20 ng/dL) at recurrence. There were no significant differences between the three groups in terms of clinical characteristics such as age, gender, tumor stage, virology status, tumor pattern, biochemical examination results, and cirrhotic status (Table 1). Significant differences between the three groups were observed for recurrence intervals, survival times and location of recurrence ($P < 0.001$, $P = 0.013$, $P = 0.001$, respectively).

Recurrence interval among the groups

The accumulated recurrence rates in the three groups were

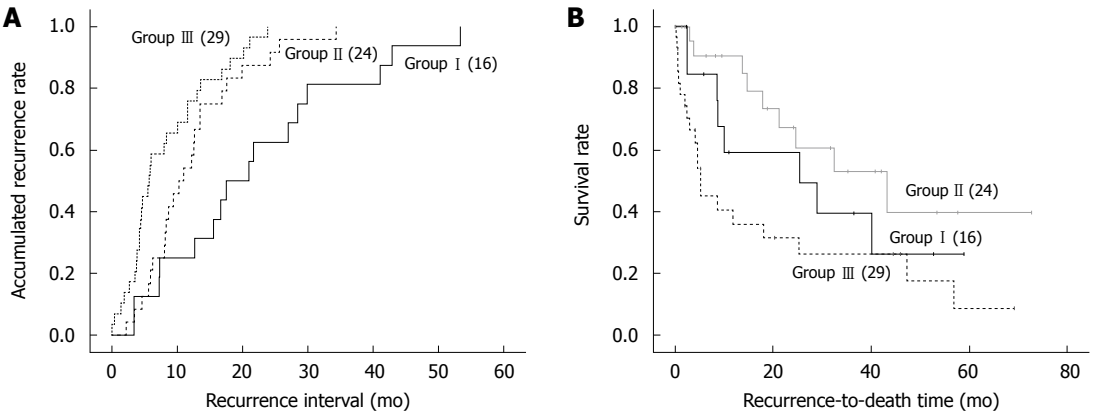


Figure 2 Accumulated recurrence rates and recurrence-to-death survival rates. A: Accumulated recurrence rates were calculated from the first liver resection date to recurrence date and compared between groups Ⅰ, Ⅱ, and Ⅲ ($P < 0.001$); B: Recurrence-to-death survival rates were calculated from the recurrence date to last follow-up date and compared between groups Ⅱ, Ⅰ, and Ⅲ ($P = 0.016$).

Table 2 Cox regression analysis of outcome for clinical parameters of HCC patients after curative liver resection

	Univariate analysis			Multivariate analysis		
	RR	95% CI	*P*	RR	95% CI	*P*
Age (yr) > 50/≤ 50	1.023	0.532-1.968	0.95			
Gender (M/F)	0.640	0.325-1.262	0.20			
Tumor size (cm) > 5/≤ 5	0.444	0.230-0.856	0.02	0.836	0.404-1.729	0.63
ALT (U/L) > 40/≤ 40	0.929	0.471-1.831	0.83			
T Bil (mg/dL) > 1.2/≤ 1.2	0.357	0.175-0.730	0.005	0.430	0.159-1.168	0.10
Albumin (g/dL) > 3.5/≤ 3.5	1.674	0.867-3.232	0.13			
Plat (10^3/μL) > 10/≤ 10	1.600	0.808-3.169	0.18			
Vessel invasion (-/+)	0.470	0.228-0.969	0.04	0.251	0.096-0.658	0.005
Tumor number (S/M)	1.073	0.556-2.070	0.83			
Child B/A	0.372	0.182-0.759	0.007	0.452	0.153-1.339	0.15
HBV (-/+)	0.598	0.262-1.363	0.22			
HCV (-/+)	1.514	0.688-3.334	0.30			
Cirrhosis (-/+)	0.885	0.452-1.734	0.72			
Group Ⅰ reference			0.005			0.04
Ⅱ/Ⅰ	0.371	0.162-0.846	0.02	2.142	0.569-7.000	0.02
Ⅲ/Ⅰ	0.318	0.144-0.700	0.004	0.582	0.254-3.033	0.12
AFP (op) (ng/dL) > 200/≤ 200	0.048	0.000-14716.4	0.64			
AFP (recur) (ng/dL) > 200/≤ 200	0.341	0.177-0.653	0.001	0.851	0.301-1.389	0.45
Recur interval (mo) > 12/≤ 12	2.550	1.265-5.145	0.009	2.438	1.112-5.348	0.03
Path grade Ⅰ reference			0.42			
Ⅱ/Ⅰ	0.821	0.376-1.795	0.62			
Ⅲ/Ⅰ	0.504	0.197-1.291	0.15			

Path grade Ⅰ, Ⅱ, Ⅲ: Histopathologic grade of HCC. Ⅰ: Well differentiated; Ⅱ: Moderately differentiated; and Ⅲ: Poor or undifferentiated.

calculated using the Kaplan-Meier method (Figure 2A). Accumulated recurrence rates were calculated from the first liver resection date to the recurrence date. Recurrence took place in all patients and was compared among groups Ⅰ, Ⅱ, and Ⅲ ($P < 0.001$). The relative ratios [95% confidence interval (CI)] of groups Ⅱ and Ⅲ compared with group Ⅰ patients were 0.244 (0.119-0.498) and 0.591 (0.340-1.027), respectively, with group Ⅰ as the reference.

Independent factors affecting survival among the groups

The risk factors for clinical outcome related to survival are shown in Table 2. The six factors affecting clinical outcome by Cox proportion hazard analysis were tumor size (> 5 cm), total bilirubin (> 1.2 mg/dL), vessel invasion (+), recurrence interval (< 12 mo), Child-Pugh classification (B), and group Ⅰ and Ⅲ. When Cox regression analysis was performed, vessel invasion, group Ⅲ, and recurrence interval were identified as independent risk factors.

Recurrence-to-death survival rates among the groups

The recurrence-to-death survival rates were calculated from the recurrence date to the last follow-up date and compared among groups Ⅰ, Ⅱ, and Ⅲ as shown in Figure 2B ($P = 0.016$). Group Ⅱ had a significantly better survival rate than the other two groups. The relative ratios (95% CI) of groups Ⅱ and Ⅲ compared with group Ⅰ were 0.550 (0.241-1.253) and 0.337 (0.153-0.743), respectively.

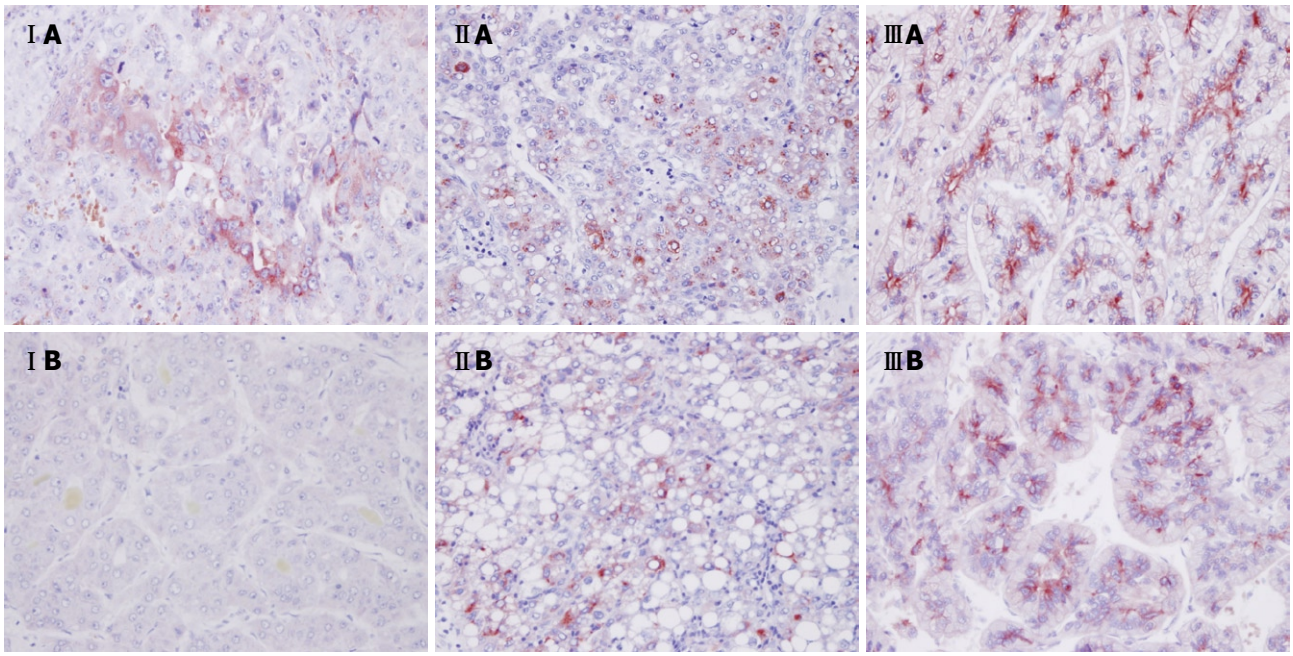


Figure 3 Immunohistochemical staining of AFP in representative specimens from the primary (A) and recurrent (B) HCC resections from each group (Ⅰ, Ⅱ, Ⅲ) × 400.

AFP immunohistochemistry in groups Ⅰ, Ⅱ, and Ⅲ

We compared the AFP immunohistochemistry results for a pair of specimens from each group. All groups revealed strong AFP staining in the primary HCC liver resection specimens. In the liver resection specimens from recurrent HCC, groups Ⅱ and Ⅲ showed strong AFP staining, but the specimen from group Ⅰ showed reduced staining (Figure 3).

DISCUSSION

Twenty-three percent of the patients in our study had inconsistent serum AFP levels between the time of initial HCC diagnosis and its recurrence. These patients had a longer recurrence interval. However, they did not have a better recurrence-to-death survival than patients with higher AFP levels at recurrence. We propose the following possible explanations: First, there was *de novo* HCC after the first resection, and the behavior or biological activity of the recurrent tumor was more malignant than the first. Second, a delay in the detection of the recurrent tumor as a result of normal AFP levels decreased the recurrence-to-death survival. This meant that these patients seemed to have a longer recurrence interval due to the delayed diagnosis caused by normal AFP levels. Once HCC recurrence was diagnosed *via* clinical symptoms, the recurrent tumor was advanced, which then shortened the time from recurrence to death.

In general, the principle we follow in HCC patients after resection is according to the NCCN guideline. For patients with initially high AFP levels, such as the patients of our study, the AFP level is followed every 3 mo, and imaging studies are carried out every 3-6 mo in the first two years. After 2 years, the AFP level is followed every 3 mo and imaging studies are carried out annually. If the AFP level is elevated, suggesting HCC recurrence then further intervention is arranged. However, in the group with inconsistent AFP levels, the imaging studies were carried out annually due to normal AFP levels. Once HCC recurrence based on the NCCN guideline is diagnosed, the recurrence will be detected late resulting in poor quality medical care and legal problems.

Histopathological change in a group Ⅰ patient and a possible mechanism

In our patients, some underwent resection twice for removal of HCC. The first and second specimens from liver resection did not show consistent histopathological findings in a patient from group Ⅰ, but did show similar histopathological findings in patients from groups Ⅱ and Ⅲ. Although these paired pathological findings are not conclusive, they do hint at an inconsistent pathological presentation. In a previous study, the authors proposed that the chromosomal changes and clonality relationship between primary and recurrent HCC could be proved using a comparative genomic hybridization technique[16]. In that study, 35% of HCC patients were considered to have a second primary HCC. In our study, the mechanism of HCC recurrence in group Ⅰ patients may be due to *de novo* HCC with low secretion of AFP. This mechanism in groups Ⅱ and Ⅲ may be due to intra-hepatic metastasis from the primary HCC. Many possible reasons for this phenomenon have been reported, including *de novo* HCC[16,17], a biologic tumor changed by dedifferentiation[18], or a multicentric origin[19].

Possible explanations for the clinical outcome

In our study, we used Cox regression analysis of the risk

factors for accumulated recurrence rate and no confounders were identified. Patients with inconsistent AFP levels had a longer accumulated recurrence rate than those in groups Ⅱ and Ⅲ. The second primary HCC might have needed a longer time to develop, resulting in a longer recurrence period. In groups Ⅱ and Ⅲ, the recurrent tumor was probably from an intra-hepatic metastasis or residual HCC resulting in a shorter recurrence interval than that suggested by other articles[20,21]. The recurrence-to-death survival rate of patients with inconsistent AFP levels was worse than that in group Ⅱ where the AFP level was consistent.

Dissociation of the tumor marker means inconsistent AFP, the clinical ramifications

Elevated serum AFP is useful for the diagnosis of HCC, and also implies a large tumor burden at presentation[6]. A low serum AFP level at recurrence in patients with an initially high AFP level at HCC diagnosis may be a good prognostic factor of survival[6]. However, this was not supported by our results. This poor outcome may be caused by a biological change in the malignancy or delayed diagnosis. Physicians need to diagnose recurrence as early as possible by regular surveillance to improve patient outcome. In the NCCN practice guidelines, version 1, 2009[15], surveillance for HCC recurrence in patients with initially elevated AFP levels includes checking AFP levels every 3 mo and performing imaging studies annually for 2 years after resection[15]. When physicians follow the NCCN guidelines, they should bear in mind that AFP level is not as sensitive as generally believed for tumor recurrence in this subgroup of HCC patients, similar to group I patients in our study. The results of our study showed that regular checks of AFP levels should be conducted every 1-3 mo, followed by imaging studies every 3 mo for patients with an initially elevated AFP level. Regular imaging studies should not be omitted even when the serum AFP level is normal. The results of our study are important for reminding practicing physicians that regular imaging is still the benchmark in the surveillance of HCC patients.

Limitations of this study

There were some limitations in this study. First, these preliminary data were from one medical center and the conclusion needs to be validated by a meta-analysis of multicentric data. Second, the diagnosis of HCC recurrence was not proven by pathologic findings. We diagnosed HCC recurrence by dynamic imaging studies, hepatic angiography, and clinical disease progression. In a further study, tumor biopsy should be performed before salvage therapy if possible. The results of histochemical staining in the recurrent tumor could explain the exact mechanism of this clinical presentation in patients with inconsistent AFP levels.

In conclusion, 23% of our selected patients demonstrated an inconsistency in serum AFP level between initial HCC diagnosis before resection and HCC recurrence diagnosis. To improve medical care and avoid missed or delayed diagnoses, regular checks of AFP level and imaging studies every three months are strongly recommended during follow-up, even for patients with normal serum AFP levels.

COMMENTS

Background

Serum α-fetoprotein (AFP) level is widely used as a tumor marker for the diagnosis and detection of both occurrence and recurrence of hepatocellular carcinoma (HCC). A high serum AFP level in HCC also indicates a poor outcome. However, inconsistent AFP levels have been found, where patients' AFP levels were initially high when HCC was diagnosed, but were normal when HCC recurred. There is no research on the prognosis of this subgroup of HCC patients.

Research frontiers

When clinical physicians follow the National Comprehensive Cancer Network guideline, they should bear in mind that AFP level is not as sensitive as generally believed for tumor recurrence in this subgroup of HCC patients. Regular checks of AFP level and imaging studies every three months are strongly recommended during follow-up, even for patients with normal serum AFP levels.

Innovations and breakthroughs

This is the first article to investigate the clinical outcomes of patients with inconsistent AFP levels. The clinical outcomes in this subgroup of HCC patients were not as good as those with normal AFP levels. To improve medical care and avoid missed or delayed diagnoses, clinical physicians should bear these findings in mind.

Applications

The authors modified the surveillance principle for this subgroup of HCC patients following the results of this study to improve the quality of medical care.

Peer review

This is a retrospective analysis of a cohort of patients with HCC, it maybe interesting for the readers.

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BRIEF ARTICLE

Combined resection and radiofrequency ablation for multifocal hepatocellular carcinoma: Prognosis and outcomes

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Abstract

AIM: To analyze the combined treatment of resection and intraoperative radiofrequency ablation (RFA) for multifocal hepatocellular carcinoma in terms of prognosis and surgical outcomes.

METHODS: This study was a retrospective case comparison study using prospectively collected data. The study covered the period from April 2001 to December 2006. The data of 200 patients with histologically confirmed hepatocellular carcinoma were reviewed. Nineteen patients (17 men and 2 women) having received resection in combination with RFA were chosen as subjects of the study (the combination group). Fifty-four patients (43 men and 11 women) having received resection alone were selected for comparison (the resection group). The two groups matched tumor number and tumor size, and all the patients in the two groups displayed no tumor rupture, major vascular involvement and distant metastasis. Their demographics, preoperative assessment, disease recurrence patterns, overall survival and disease-free survival were compared.

RESULTS: In the combination group, the median age was 65 years (range, 34-77 years), the median tumor number was 3 (range, 2-9), and the median tumor size was 6 cm (range, 1.2-14 cm). In the resection group, the median age was 51.5 years (range, 27-80 years, P = 0.003), the median tumor number was 3 (range, 2-9, P = 0.574), and the median tumor size was 6 cm (range, 1-14 cm, P = 0.782). The two groups were similar in characteristics of tumors and comorbidities, and had comparable results in preoperative liver function tests. All patients had Child-Pugh class A status. Bilobar involvement occurred in 14 patients (73.6%) in the combination group and 3 patients (5.5%) in the resection group (P = 0.04). Six patients (32%) in the combination group and 35 patients (65%) in the resection group underwent major hepatectomy. Thirteen patients (68%) in the combination group and 19 patients (35%) in the resection group underwent minor hepatectomy (P = 0.012). The combination group had fewer major resections (32% *vs* 65%, P = 0.012), less blood loss (400 *vs* 657 mL, P = 0.007), shorter operation time (270 *vs* 400 min, P = 0.001), and shorter hospital stay (7 *vs* 8.5 d, P = 0.042). The two groups displayed no major differences in surgical complications (15.8% *vs* 31.5%, P = 0.24), disease recurrence (63.2% *vs* 50%, P = 0.673), hospital mortality (5.3% *vs* 5.6%, P = 1), and overall survival (53 *vs* 44.5 mo, P = 0.496).

CONCLUSION: Safe and effective for selected patients with multifocal hepatocellular carcinoma, the combination of resection and intraoperative RFA widens the applicability of surgical intervention for the disease.

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Key words: Hepatocellular carcinoma; Radiofrequency ablation; Combined resection; Resection; Cirrhosis

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INTRODUCTION

Management of hepatocellular carcinoma (HCC) in patients with cirrhosis is always a great challenge for clinicians. Prognosis of most HCC patients is poor due to the low rate of tumor resectability (20%-37%)[1,2]. Although resection is regarded as the gold-standard treatment for HCC, many patients are not suitable for it because of unfavorable anatomical location, major vessel involvement, multifocal involvement, distant metastasis, or poor liver function[3-7]. In the hope of improving the survival of patients having unresectable HCC, locoregional treatment aiming at local tumor control has been developed.

Radiofrequency ablation (RFA) is a locoregional treatment which has gained recognition in the management of liver diseases including HCC. During RFA treatment, heat energy generated by high-frequency alternating currents (460-480 kHz) targeted at the living tissues causes protein denaturation at a temperature of 60℃ through ionic vibration; coagulative necrosis of the target lesion then follows. RFA outperforms other locoregional treatments by its effective tumor ablation, minimal liver damage, low morbidity, and low mortality[3,8-12].

Lately, there have been detailed studies on RFA treating small HCC as well as isolated reports on the combined employment of resection and RFA for the treatment of liver metastasis of HCC[13-15]. Curley *et al*[16] reported a series of RFA treatment on 123 patients bearing unresectable primary (n = 48) or metastatic (n = 75) liver tumors. A total of 169 tumors were ablated with a clearance rate of 100%. There was no operative mortality. However, the efficacy of RFA on large tumors is still questionable. Although several reports showed that RFA with multiple processes and overlap of ablation zones was feasible for treating large liver tumors, the high recurrence rates did not favor it as a primary treatment tool for them[17-22]. Resection is still considered the first treatment option for large HCC.

Patients with multiple HCCs can opt for multiple resections, but multiple resections entail a higher risk of liver failure for patients with cirrhosis. Hence, in the management of multifocal HCC, combined employment of resection (for large tumors) and RFA (for small tumors) sounds like a reasonable measure for the achievement of complete tumor clearance.

Both resection and RFA are good treatment options for HCC while at the same time they both have their own limitations. Resection is limited by the patient's liver function and the location of tumor, and RFA is limited to small tumors only. In general, around one-fourth of patients with HCC are amenable to surgical intervention, and among them, around one-fourth develop multiple tumors on presentation. The combination of resection and RFA can subject more patients to potentially curative surgical intervention.

This study compared the combined treatment of resection and RFA with conventional hepatectomy alone in terms of prognosis and surgical outcomes in treating multifocal HCC.

MATERIALS AND METHODS

This study was a retrospective case comparison study using prospectively collected data. In our hospital, the data of all patients of liver surgery are input into a detailed computerized database by the surgeon-in-charge after patient discharge. Data collected include demographics, preoperative investigation results, operative details, and postoperative investigation results. This study covered the period from April 2001 to December 2006. The data of 200 patients with histologically confirmed HCC were reviewed. Nineteen patients having received resection in combination with RFA were chosen as subjects of the study (the combination group). Fifty-four patients having received resection alone were selected for comparison (the resection group). The two groups matched in terms of tumor number (range, 2-9) and tumor size (within 14 cm), and all patients in the two groups displayed no tumor rupture, major vascular involvement and distant metastasis. Their demographics, preoperative assessment, disease recurrence patterns, overall survival and disease-free survival were compared.

All the 73 patients had their medical history reviewed and were fully assessed with physical examination. They received chest X-ray (CXR) and computed tomography (CT) scan of the abdomen. They also received serum laboratory tests which yielded information on complete blood count, platelet count, coagulation profile, hepatitis B and hepatitis C serology, total serum bilirubin, serum albumin, serum alanine aminotransferase, serum aspartate aminotransferase, serum gamma-glutamyl transferase, serum alkaline phosphatase, serum α-fetoprotein (AFP), electrolytes, renal function, and indocyanine green (ICG) clearance rate. Resectability of tumor was confirmed upon three criteria: first, there was no distant metastasis according to the results of CXR and CT scan; second, the ICG rate was less than 14.4% at 15 min; and third, the estimated mass volume of the residual liver after resection was more than 30% of the original liver.

Surgical approaches

Each patient in the combination group underwent resection and RFA in one single operation intended to cure. The operation was performed with the open approach, starting with an initial exploration of the abdomen and pelvis to confirm the absence of extrahepatic lesion.

Intraoperative ultrasound was used to identify tumor location and number as well as the relation between the tumors and the vasculature of the liver. Anatomical resection was performed for the largest tumors or the largest groups of tumors which were deemed surgically resectable with clear margins. The extent of resection was determined by lesion location, the relation between the lesion and the surrounding vasculature, and biliary involvement. Parenchymal transection was performed with a Cavitron ultrasonic surgical aspirator. Segmental pedicles and other major vasculatures were divided with vascular staplers or ligatures. Pringle's maneuver was not applied.

Lesions not treated with resection were then subjected to RFA with standard treatment algorithm. Intraoperative ultrasound was used to guide the placement of the RFA needle at the target lesion. Single needle or needle cluster was used according to the size of the target tumor. For tumors larger than 3 cm, cluster probe was used. A margin of 1 cm was included in ablation. The Cool-tip™ system which consisted of a generator that supplied power up to 200 W was used. The electrode was optimally positioned to achieve complete tumor destruction and a normal parenchymal ablation zone of at least 1 cm.

All the patients were originally enlisted for resection only, but due to various reasons, the operative surgeon adopted RFA as an additional treatment modality with curative intention for complete control of tumors. The reasons for the adoption of a combination therapy are listed in Table 1.

Table 1 Reasons for adopting combination therapy *n* (%)

	Combination group (*n* = 19)
Bilobar disease	14 (73.6)
Proximity to major vessel or bile duct	5 (26.3)
Dense adhesion	3 (15.8)
Large resection required for small tumors	5 (26.3)
ICG rate at 15 min > 14.4%	5 (26.3)
Low platelet count (< 100 × 10^9/L)	3 (15.8)
Severe cirrhosis	9 (47.4)

ICG: Indocyanine green.

Table 2 Patient demographics

	Combination group (*n* = 19)	Resection group (*n* = 54)	*P* value
Age (yr)	65 (34-77)	52 (27-80)	0.003
Gender (M:F)	17:2	43:11	0.492
Comorbidities	10 (53%)	20 (37%)	0.235
Renal impairment	0 (0%)	2 (3.7%)	1.000
Diabetes mellitus	5 (26.3%)	10 (18.5%)	0.469
Chest infection	1 (5.3%)	3 (5.6%)	1.000
Coronary complications	6 (31.6%)	14 (25.9%)	0.635
Hepatitis B infection	16 (84.2%)	42 (77.8%)	0.745
Hepatitis C infection	2 (10.5%)	2 (3.7%)	0.478
Child-Pugh class A	19	54	1.000
Platelet count (× 10^9/L)	165 (91-64.5)	177 (86-458)	0.396
ICG (% at 15 min)	11.8 (3-25.7)	9 (3.7-18.2)	0.083
AFP level (ng/mL)	248 (6-38 040)	133 (2-530 600)	0.915
Tumor size (cm)	6 (1.2-14)	6 (1-12.5)	0.782
Tumor number	3 (2-9)	3 (2-9)	0.574

AFP: α-fetoprotein.

Postoperative follow-up

Monitoring of complete blood picture, coagulation profile, blood gas, liver function and renal function was carried out on day 1 and day 7 as part of the standard protocol. ICG clearance test was done on day 7 if possible. CXR, CT scan of the abdomen and serum AFP investigation were performed at one month, then quarterly in the first two years, and half-yearly afterwards.

Statistical analysis

All clinical data were analyzed with SPSS version 11.5 under the Window 98 operating system. Categorical variables were compared by a χ^2 test or Fisher's exact test. Continuous variables were compared by the Mann-Whitney *U* test. Survival rates were calculated by the Kaplan-Meier method, and differences in survival were analyzed by the log rank test. *P* value < 0.05 was considered statistically significant. The primary outcome measurement was overall survival and the secondary outcome measurement was disease-free survival. Immediate postoperative performance was also analyzed.

RESULTS

In the combination group, 17 men and 2 women, the median age was 65 years (range, 34-77 years). The median tumor number was 3 (range, 2-9) and the median tumor size was 6 cm (range, 1.2-14 cm). In the resection group, 43 men and 11 women, the median age was 51.5 years (range, 27-80 years, $P = 0.003$). The median tumor number was 3 (range, 2-9, $P = 0.574$) and the median tumor size was 6 cm (range, 1-14 cm, $P = 0.782$). The two groups were similar in characteristics of tumors and comorbidities, and had comparable results in preoperative liver function tests on serum bilirubin, serum albumin, prothrombin time, platelet count and ICG clearance. All patients in the two groups had Child-Pugh class A status (Table 2). Bilobar involvement occurred in 14 patients (73.6%) in the combination group and 3 patients (5.5%) in the resection group ($P = 0.04$).

Six patients (32%) in the combination group and 35 patients (65%) in the resection group received major hepatectomy. Thirteen patients (68%) in the combination group and 19 patients (35%) in the resection group received minor hepatectomy ($P = 0.012$). The types of resection performed are listed in Table 3. In the combination group, 5 patients had small tumor but received a large volume of resection for clearance, and 5 patients had tumors located close to major vessel, bile duct, or junction of hepatic veins. A total of 31 tumors were ablated, and the median size of the tumors was 1 cm (range, 1-3.8 cm). Ten patients had 1 tumor ablated, 6 patients 2, and 3 patients 3. Biopsies of the ablation sites were

Table 3 Types of resection performed according to Brisbane terminology (2005) of liver resection *n* (%)

	Combination group (*n* = 19)	Resection group (*n* = 54)
Right hepatectomy	1 (5.3)	17 (31.5)
Extended right hepatectomy	0 (0)	6 (11.1)
Right trisectionectomy	0 (0)	2 (3.7)
Left hepatectomy	3 (15.8)	3 (5.6)
Extended left hepatectomy	2 (10.5)	4 (7.4)
Left trisectionectomy	0 (0)	3 (5.6)
Left lateral sectionectomy	3 (15.8)	1 (1.9)
Segmentectomy	1 (5.3)	11 (20.4)
Wedge resection of liver	9 (47.4)	7 (13)

taken and all proven to be positive for HCC. The ablated sites were monitored with CT scan with contrast at one month, and no incomplete ablation was noted.

The combination group demonstrated certain advantages over the resection group in terms of surgical outcomes: the former had median blood loss of 400 mL (range, 20-1650 mL) whereas the latter had 657 mL (range, 50-3750 mL) (P = 0.007), the former had median operation time of 270 min (range, 150-465 min) whereas the latter had 400 min (range, 165-773 min) (P = 0.001), and the former had median hospital stay of 7 d (range, 1-20 d) and the latter had 8.5 d (range, 4-69 d) (P = 0.042). There was no difference in overall complications (15.8% *vs* 31.5%, P = 0.241) and hospital mortality (5.3% *vs* 5.6%, P = 1) between the two groups. Only one patient from the resection group needed blood transfusion. The median serum bilirubin level at day 1 was 21 μmol/L (range, 10-37 μmol/L) in the combination group and 25 μmol/L (range, 9-110 μmol/L) in the resection group (P = 0.012). The median serum albumin level at day 1 was 31.5 g/L (range, 10-37 g/L) in the combination group and 32 g/L (range, 17-39 g/L) in the resection group (P = 0.215). The median serum bilirubin level at day 7 was 18 μmol/L (range, 11-27 μmol/L) in the combination group and 21 μmol/L (range, 3-190 μmol/L) in the resection group (P = 0.075). The median serum albumin level at day 7 was 30 g/L (range, 24-39 g/L) in the combination group and 32 g/L (range, 26-41 g/L) in the resection group (P = 0.036).

Histopathology

With regard to the pathology of tumors, the combination group and the resection group shared similar characteristics. Margin involvement happened on none of the patients in the former group while on 4 patients (7.4%) in the latter (P = 0.567). Vascular permeation happened on 7 patients (36.8%) in the former group and 31 patients (57.4%) in the latter (P = 0.123). Microsatellite lesions appeared in 3 patients (15.8%) of the former group and 14 patients (25.9%) of the latter (P = 0.53). Poorly differentiated cell types by Edmondson classification appeared in 2 patients (10.5%) of the former group and 8 patients (14.8%) of the latter (P = 0.857). There was no statistical difference in histopathology between the two groups (Table 4).

Table 4 Histopathological results of tumors *n* (%)

	Combination group (*n* = 19)	Resection group (*n* = 54)	*P* value
Margin involvement	0 (0)	4 (7.4)	0.567
Vascular permeation	7 (36.8)	31 (57.4)	0.123
Microsatellite lesion	3 (15.8)	14 (25.9)	0.530
Poorly differentiated cell type	2 (10.5)	8 (14.8)	0.857
Complete ablation	19 (100)	-	
Histological proof of HCC at ablation sites	19 (100)	-	

HCC: Hepatocellular carcinoma.

Recurrence

Twelve patients (63.2%) in the combination group were found developing recurrence at the end of the study. Intrahepatic recurrence occurred in 8 patients (42.1%), and among them 4 patients (20.5%) had ablation site local recurrence, which happened at a median time of 3.6 mo (range, 3-24 mo). Extrahepatic recurrence occurred in 1 patient (5.3%) and intrahepatic and extrahepatic recurrence in 3 (15.8%). In the resection group, 27 patients (50%) were found developing recurrence. Among them, 15 patients (27.8%) had intrahepatic recurrence, 4 patients (7.4%) extrahepatic recurrence, and 8 patients (14.8%) intrahepatic and extrahepatic recurrence (P = 0.673).

Survival

The median follow-up period was 18.4 mo for the entire study period, 21 mo (range, 0.03-53 mo) for the combination group and 15.1 mo (range, 0.37-68.4 mo) for the resection group. No patient was lost to follow-up. The rates of overall survival at 1 year and 3 years were 88.8% and 62.6%, respectively for the former group (median 53 mo), and 88.9% and 51.8%, respectively for the latter (median 44.5 mo) (P = 0.496) (Figure 1A). The median disease-free survival of the former was 8.8 mo and that of the latter was 9.8 mo (P = 0.64) (Figure 1B). The two groups showed no statistically significant differences in survival.

DISCUSSION

A variety of treatment options are available for HCC. Liver transplantation offers good hope of cure to HCC patients, even those with poor liver function. However, donated livers are scarce, and patients having tumors exceeding the size limit for liver transplant or having low score in Model for End-stage Liver Disease are not suitable for transplantation. So, the first-line option is still, if technically feasible, hepatectomy[1,4,23,24]. Hepatectomy with clear margin can provide long postoperative survival to patients. Unfortunately, poor liver function poses extra challenge to hepatectomy because hepatectomy will diminish the already limited liver function reserve, thus risking liver failure. In Southeast Asia including Hong Kong, HCC patients usually have poor liver function because their HCC often coexists with hepatitis B cirrhosis.

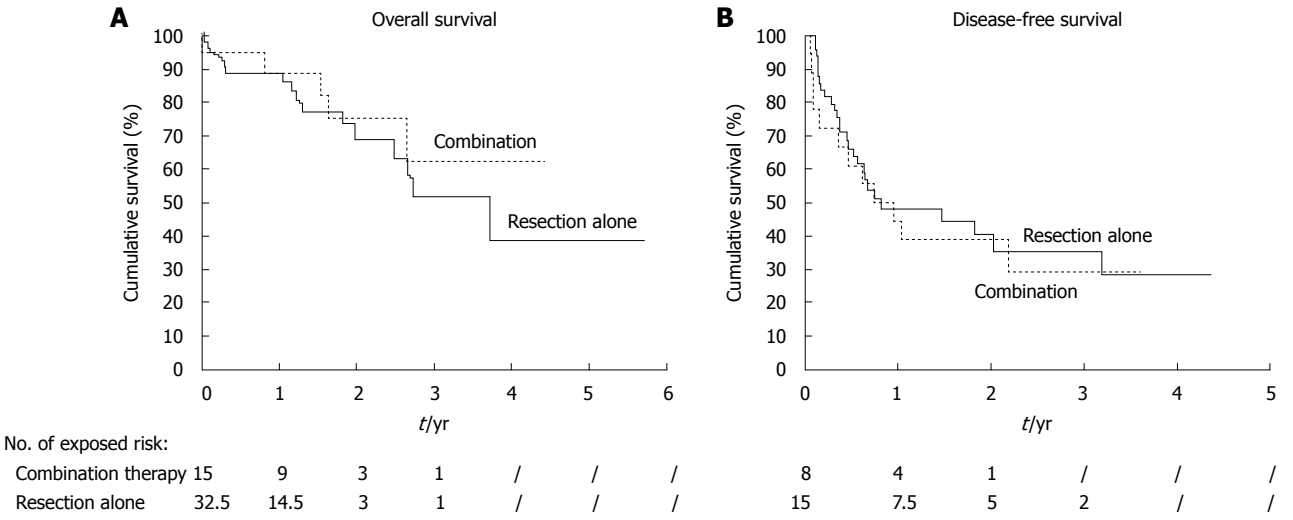


Figure 1 Survival curves showing overall survival and disease-free survival of the two groups of patients. A: Overall survival of patients; B: Disease-free survival of patients.

Striking a balance between achieving adequate resection and preventing liver failure is not easy at all. In patients with multifocal HCC, equilibrium between complete tumor clearance and minimal hepatectomy volume is even more difficult to grasp. Multiple partial resections, on the one hand, can achieve complete tumor clearance but, on the other hand, carry a higher risk of liver failure with doubtful long-term oncological outcomes.

RFA is another treatment option for HCC. Several clinical trials showed that RFA was as effective as hepatectomy for HCC smaller than 5 cm with a morbidity rate of less than 15%[22,25,26]. Hence, RFA has been gaining recognition in treating liver cancer including advanced HCC. Nonetheless, the efficacy of RFA on larger HCC is still questionable. In a study using a porcine model, RFA of 30%-35% of liver and hepatectomy of the same volume were compared, and it was found that the former resulted in significantly adverse systemic inflammatory response syndrome[27-29]. Hoshida *et al*[30] showed in another study that large parenchymal RFA volume was an independent poor prognostic factor for HCC patients. With recent advances in the design of radiofrequency electrodes and refinements of ablation techniques, favorable outcomes of RFA treatment on large tumors have been reported. However, extra caution should always be taken in treating large tumors with RFA since ablation of large tumors is associated with a high incidence of local recurrence, and the large amount of necrotic tissue left behind can cause serious problems for the patients. To ensure better outcome of RFA, careful patient selection, meticulous techniques and close monitoring of hepatic and renal functions after the procedure are essential.

Combined employment of hepatectomy and RFA for metastatic liver cancer has been documented. This combination of treatments can improve the tumor resectability rate for patients having multiple tumors. In this comparatively novel treatment, large tumors are resected and smaller ones at difficult locations are ablated. Currently, data on its performance in the management of HCC are very limited[14-16,22,25,26,31]. In our present study, the surgical outcomes of the combined treatment of hepatectomy and RFA on patients with multifocal HCC were investigated, and it was shown that patients having bilobar HCC could be treated safely with it. In the study, considerably less major hepatectomies were performed for the combination group, yet complete tumor clearance was still achieved. This is of crucial importance as hepatectomy in a lesser extent means better preservation of liver reserve, less liver failure, and quicker recovery[32,33].

For managing multifocal HCC, one may argue that multiple anatomical resections can achieve similar results as the combination approach. But in our study, we found that there are situations in which the combination approach is easier, if not better, than resection alone. First of all, if a tumor is small but requires a large resection volume for tumor clearance, RFA comes in as a safer option in terms of liver function preservation. Secondly, if a tumor is close to an important anatomical structure such as major vessels or bile ducts, RFA together with bile duct cooling can offer complete tumor clearance, obviating major hepatectomy. Thirdly, in the case of reoperation at a site where there is dense adhesion, RFA is a less traumatic treatment option, as mobilization for resection may lead to bleeding problem, especially in patients with thrombocytopenia and coagulopathy.

In this study, the median age of the combination group was 65 years, which was 13 years older than the 52-year median of the resection group. Choice of treatment was up to individual surgeons. It turned out that more elderly patients received the combined treatment. This was probably because the combined treatment would require less operation time and induce seemingly less surgical trauma. The resultant shorter operation time, less blood loss and shorter hospital stay have vindicated the postulation.

An ablation rate of 100% was achieved as confirmed by CT scan of the abdomen performed one month after

RFA treatment. The two groups displayed similar recurrence patterns. A margin of 1 cm was sought in all resections and ablations since a 1-cm margin is usually effective in preventing microscopic involvement by the major tumor. However, a clear margin alone is not enough for preventing tumor recurrence, as apart from margin involvement, there are other factors affecting recurrence such as poorly differentiated cell type, lymphovascular permeation, and microsatellite lesion, among which the latter two are the most significant independent risk factors. Unfortunately, we cannot alter these factors even by providing generous margins[24].

The combination group had more bilobar cases than the resection group (73.6% *vs* 7.4%). Theoretically, patients with stage IVa HCC would fare worse[34,35]. The predominance of bilobar disease in the combination group also explains why the group had more, though not significantly, intrahepatic and extrahepatic recurrences. A more advanced stage of HCC probably entails a poorer prognosis in general. Sub-group analysis of overall survival of these patients in the two groups was performed, and the median survival was 53 mo in the combination group and 44.5 mo in the resection group ($P = 0.496$). No significant difference was noted because of the small sample number in the resection group ($n = 3$). In our hospital, the median survival of patients having stage IVa HCC is 10 mo if they undergo no treatment. In the past, bilobar liver disease was considered a contraindication to hepatectomy, but with new tools and techniques, the whole concept of resectability is changing. Liu *et al*[36] pointed out that resection offered better survival outcomes than non-resectional treatments did to HCC patients with stage IVa bilobar disease. For this category of patients, resection in combination with RFA is an attractive choice of treatment. As to survival in our present study, the combination group and the resection group displayed comparable rates of overall survival and disease-free survival.

This study has clearly shown that when complete resection by major hepatectomy is dangerous because of marginal liver function or difficult tumor location, selective use of RFA is helpful. The integration of RFA into resectional surgery contributes to complete removal of tumors with adequate margin, diminishes the extent of parenchymal resection, and improves the resectability rate for patients with stage IVa liver disease. Safe and quick, it is especially handy when unexpected tumor is discovered during laparotomy, allowing complete tumor clearance in an unplanned situation during surgical operation without altering the original plan of treatment procedure too much; this is particularly important to patients having marginal hepatic function.

Fresh hope emerges as tumors previously considered unresectable due to multifocal involvement or poor liver function reserve can now be removed by the combined treatment, a new option in treating HCC. At present, a randomized controlled trial comparing it with surgical resection alone in patients with multifocal HCC is much desirable.

COMMENTS

Background

Management of hepatocellular carcinoma (HCC) in patients with cirrhosis is always a great challenge for clinicians. Prognosis of these patients is poor because many of their tumors are unresectable (with a resectability rate of 20%-37% only). Although resection is regarded as the gold-standard treatment for HCC, it is not applicable to cases with unfavorable anatomical location, major vessel involvement, multifocal involvement, distant metastasis, or poor liver function. On the other hand, radiofrequency ablation (RFA) is a treatment which has been gaining more and more recognition in the management of liver diseases including HCC. Both resection and RFA are good treatment options for HCC. However, they have their own limitations. Resection is hampered by patient's liver function as well as the location of tumor, whilst RFA is limited to smaller tumors only. In reality, only around one-fourth of HCC patients are amenable to surgical intervention, and in this sub-group, around one-fourth develop multiple tumors on presentation. There is no doubt that the combination of resection and RFA can subject more patients to potentially curative surgical intervention. The present study compared the combined treatment of resection and RFA with the treatment of sole resection and investigated the surgical outcomes of the combined treatment in patients with multifocal HCC.

Research frontiers

There is no detailed report on the combined use of resection and RFA in the management of HCC. However, this is an important topic as treating HCC with RFA is getting popular, and combining RFA with resection can definitely extend the operability of surgical intervention to patients with multifocal HCC.

Innovations and breakthroughs

In the management of multifocal HCC, one may argue that multiple resections can achieve what the combined treatment achieves, and thus there is no need to call in RFA. However, in the present study, it has been found that in some situations the combined treatment is easier than resection alone. First of all, since RFA is less traumatic and hence better for liver function preservation, it is preferable to resection when the tumor is small but still requires a large volume of resection for tumor clearance. Secondly, when the tumor is close to an important anatomical structure such as major vessel or bile duct, RFA together with bile duct cooling can achieve complete tumor clearance with lower risk. Thirdly, if dense adhesion is encountered, which is not uncommon in re-operation, RFA is safer as mobilization of the liver for resection may lead to bleeding problem especially in patients with thrombocytopenia and coagulopathy.

Applications

This study has clearly shown that when complete resection by major hepatectomy is dangerous because of marginal liver function or difficult tumor location, selective use of RFA provides the benefit of increased tumor resectability. The integration of RFA into resectional surgery contributes to complete removal of tumors with adequate margin, diminishes the extent of parenchymal resection, and improves the resectability rate for patients with stage IVa liver disease. Being safe and quick, it is especially handy when unexpected tumor is encountered during laparotomy, allowing achievement of complete tumor clearance in an unplanned situation during surgical operation without altering the original plan of treatment procedure too much; this is particularly important to patients with marginal liver function.

Terminology

RFA is a locoregional treatment. RFA treats tumors by inducing coagulative necrosis of the target lesion. During RFA, heat energy generated by high-frequency alternating currents (460-480 kHz) targeted at the lesion through ionic vibration causes protein denature at a temperature of 60℃, which causes coagulative necrosis of tissues.

Peer review

The Hong Kong group describes their experience with surgical treatment of HCC by performing a retrospective analysis of resected-only patients versus resected + RFA patients with HCC. This is important data to present and very clinically relevant as centers all over the world are taking care of rising numbers of patients with HCC.

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BRIEF ARTICLE

Evidence-based appraisal in laparoscopic Nissen and Toupet fundoplications for gastroesophageal reflux disease

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Abstract

AIM: To demonstrate the optimal surgical procedure for gastroesophageal reflux disease.

METHODS: The electronic databases of Medline, Elsevier, Springerlink and Embase over the last 16 years were searched. All clinical trials involved in the outcomes of laparoscopic Nissen fundoplication (LNF) and laparoscopic Toupet fundoplication (LTF) were identified. The data of assessment in benefits and adverse results of LNF and LTF were extracted and compared using meta-analysis.

RESULTS: We ultimately identified a total of 32 references reporting nine randomized controlled trials, eight prospective cohort trials and 15 retrospective trials. These studies reported a total of 6236 patients, of whom 4252 (68.18%) underwent LNF and 1984 (31.82%) underwent LTF. There were no differences between LNF and LTF in patients' satisfaction, perioperative complications, postoperative heartburn, reflux recurrence and re-operation. Both LNF and LTF enhanced the function of lower esophageal sphincter and improved esophagitis. The postoperative dysphagia, gas-bloating syndrome, inability to belch and the need for dilatation after LNF were more common than after LTF. Subgroup analyses showed that dysphagia after LNF and LTF was similar in patients with normal esophageal peristalsis (EP), but occurred more frequently in patients with weak EP after LNF than after LTF. Furthermore, patients with normal EP after LNF still had a higher risk of developing dysphagia than did patients with abnormal EP after LTF.

CONCLUSION: Compared with LNF, LTF offers equivalent symptom relief and reduces adverse results.

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Key words: Laparoscopic fundoplication; Nissen, Toupet; Gastroesophageal reflux disease; Anti-reflux surgery; Esophageal peristalsis; Meta-analysis

Peer reviewer: Marcelo A Beltran, MD, Chairman of Surgery, Hospital La serena, PO Box 912, La Serena, IV Region, Chile

Shan CX, Zhang W, Zheng XM, Jiang DZ, Liu S, Qiu M. Evidence-based appraisal in laparoscopic Nissen and Toupet fundoplications for gastroesophageal reflux disease. *World J Gastroenterol* 2010; 16(24): 3063-3071 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i24/3063.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i24.3063

INTRODUCTION

Gastroesophageal reflux disease (GERD) is a chronic acid-peptic disorder characterized by the spontaneous and involuntary retrograde flow of stomach contents into esophagus mainly due to functional defect of the lower esophageal sphincter (LES)[1]. Acid reflux into the esophagus may cause esophagitis, tracheobronchitis,

stricture, Barrett's esophagus and even esophageal cancer, all of which affect a sizeable portion of patients, particularly in the USA and Europe[2-5]. Symptomatic GERD has fundamental effects on the quality-of-life (QOL) of patients. However, the complex pathophysiology of GERD[4] and limitation of the medicine[6] always make it extremely difficult to get a satisfactory efficacy under individual anti-acid medication in the long term.

Surgery is discussed as a very effective treatment for GERD which imparts a mechanical solution[6]. Surgical treatment was initially introduced by Rudolph Nissen[7] in 1956 and subsequently was developed as a safe and effective procedure by Dallemagne in 1991 through a minimal invasive approach[8]. Laparoscopic fundoplication has been established as the "gold standard" in the surgical treatment for GERD because of its immense success. There are two major anti-reflux procedures: 360º total (Nissen) fundoplication and 270º partial (Toupet) fundoplication. The superiority of one over the other is a matter of debate. The supporters of partial fundoplication argue that partial and total wrap construction offer equally effective forms of therapy for GERD[9-11]. To avoid the major postoperative complication, dysphagia, preoperative esophageal motility should be considered, and partial funduplication has the advantage of reducing this complication[12,13]. The proponents of total fundoplication propose that: (1) the 270º wrap provides a weaker anti-reflux barrier, and is therefore insufficient for reflux control[14,15]; (2) the prevalence of postoperative dysphagia after Nissen fundoplication is overestimated; and (3) motility disorders are not correlated with postoperative dysphagia[16], suggesting that the so-called "tailored procedure" should be abandoned[17,18]. Many crucial issues related to the mechanism of GERD, such as whether esophageal dysmotility is a consequence of GERD or just another component (along with LES dysfunction) of a complex foregut motor disease, have not been elucidated[19,20], so the controversy regarding the optimal surgical technique continues[21,22].

Meta-analysis is a powerful tool with an attempt to overcome the problem of reduced statistical power in studies with small sample sizes and to control variations between studies. The goal of surgical treatment for GERD is to provide optimal reflux control while minimizing adverse results. We focused this current systemic review on comparing which procedure was more effective in improving the QOL of patients while producing less adverse results. We attempted to depict a more comprehensive picture of the feature of laparoscopic anti-reflux operation, thus offering guidance for clinical practice.

MATERIALS AND METHODS

Search strategy

The electronic databases of Medline, ScienceDirect (Elsevier), Springerlink and Embase over the last 16 years from January 1994 to November 2009 were searched by two authors (Shan CX and Zhang W) to identify all clinical trials comparing laparoscopic Nissen fundoplication (LNF) with laparoscopic Toupet fundoplication (LTF). A search strategy using disease-specific search terms (e.g. gastroesophageal reflux disease), management-specific terms (e.g. laparoscopic anti-reflux fundoplication) and terms related to surgical procedures (e.g. Nissen, Toupet, partial and total) was adopted. All photocopied abstracts and citations were reviewed. The related articles were used to broaden the searching scope, and references of the articles acquired were also searched.

Selection criteria

Abstracts or full-text articles were initially screened, and then selected or rejected by the two reviewers (Shan CX and Zhang W) on the basis of the inclusion and exclusion criteria described below. A flow chart representing selection of clinical trials is shown in Figure 1.

Inclusion criteria: (1) Comparative clinical trials, including randomized and non-randomized controlled trials, involved in the efficacy and adverse results of different types of laparoscopic anti-reflux surgery (LARS); and (2) The exact data of dichotomous-type information and continuous-type information as well as standard deviation should be provided so as to integrate each single weight in each study.

Exclusion criteria: (1) Comparative trials between total and non-posterior partial fundoplication (e.g. total *vs* anterior partial fundoplication); (2) Fundoplications were carried out with laparotomy; (3) Trials involving children or patients younger than 16 years; and (4) Studies published repeatedly by different journals.

Data extraction

The two reviewers independently extracted details from selected studies which comprised (1) information and quality of the research: first author, year of publication, comparative design, sample capacity, follow-up duration; and (2) outcome analysis, including beneficial and adverse results. The specific rules of data extraction were listed below.

First, the assessment data of repeated trials published in different journals at different phases was extracted based on the latest article. For example, the results in the studies of Strate *et al*[16], Fibbe *et al*[23] and Zornig *et al*[24] were highly homologous, containing identical study objects and design protocol. Thus, the source of data extraction was focused on the latest citation of Zornig *et al*[24].

Second, the assessment data of trials containing multiple groups were initially divided into single groups, and then extracted individually. For example, the research of Mickevicius *et al*[22] included two subgroups of LARS with a 1.5-cm wrap fundoplication and 3.0-cm wrap. We split it into two independent trials according to the wrap length.

And third, the evaluation indices of surgical efficacy and incidence of postoperative morbidities were subject to the final updates based on long-term follow-up results. For example, Chrysos *et al*[25] reported the 3- and 12-mo incidences of postoperative dysphagia in their

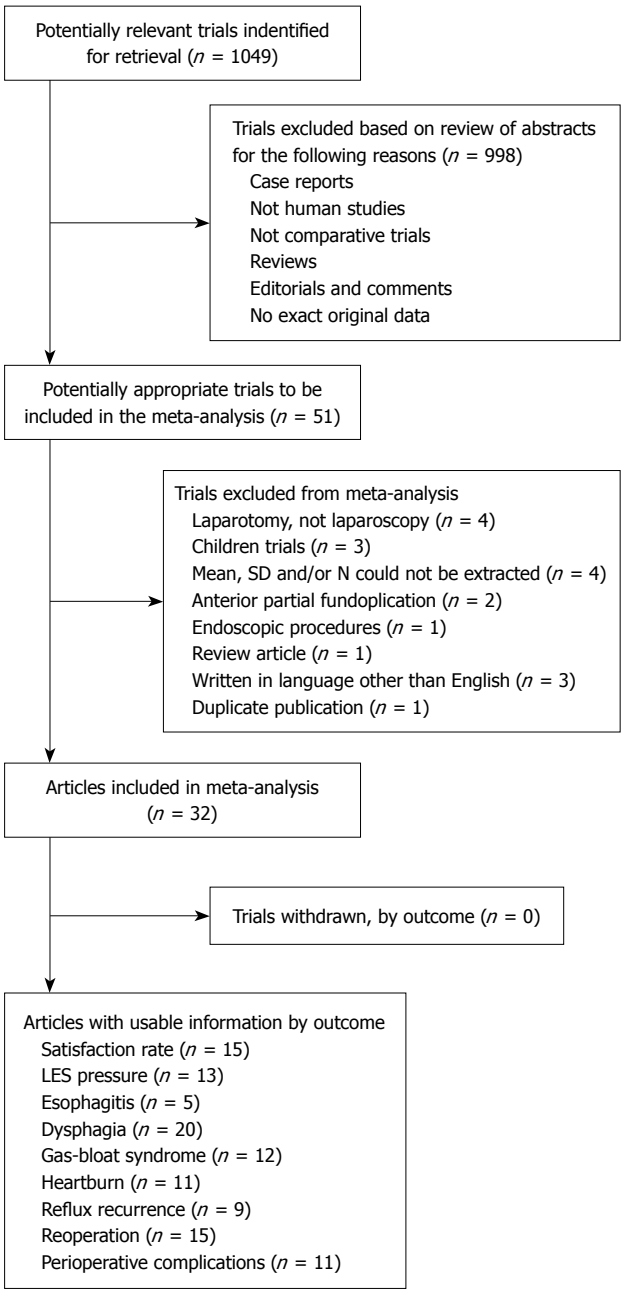


Figure 1 A flow chart showing the progress of trials through the review. LES: Lower esophageal sphincter.

article. We ultimately extracted the information on the 12-mo incidence of dysphagia after LARS and integrated it into the total meta-analysis.

Statistical analysis

All individual outcomes were integrated with the meta-analysis software: Review Manager Software 5.0 (Cochrane Collaborative, Oxford, England). Results were analyzed with the random-effect method if significant heterogeneity ($P < 0.05$ was used to define statistically significant heterogeneity) was detected among studies. Otherwise, a fixed-effect model was adopted. The odds ratio (OR) and the weighted mean difference were calculated for dichotomous data and continuous data, respectively. In addition, subgroup analyses were done to estimate if the results of postoperative dysphagia would change after LARS with respect to preoperative esophageal motility (EM). Thus, three subgroups were established. Subgroup 1: all patients underwent LNF or LTF and had normal esophageal peristalsis (EP); subgroup 2: all patients underwent LNF or LTF and had abnormal EP; and subgroup 3: LNF for patients with normal EP *vs* LTF for patients with abnormal EP.

RESULTS

Identification and characteristics of studies and patients

We ultimately identified 32 references reporting 29 independent controlled studies published between January 1994 and November 2009. Nine randomized controlled trials[10,16,21-27], eight prospective cohort trials[11,17,28-33] and 15 retrospective trials[9,12,20,33-45] met the selection criteria reporting 6236 patients, of whom 4252 (68.18%) underwent LNF and 1984 (31.82%) underwent LTF. Twenty studies provided the sex and 11 trials provided the age of included patients. The percentage of males varied from 39.39% to 70.13% in the LNF group, and from 39.53% to 66.67% in the LTF group. The mean age ranged from 45.2 to 59.2 years in the LNF group and from 44.2 to 61.7 years in the LTF group. The details of these studies are shown in Table 1.

Subjective assessment: Satisfaction rate

Fifteen studies assessed patient satisfaction as the major efficacy of LARS. In addition, patient satisfaction with the outcome of surgery was always expressed by Visick scores evaluating QOL. After follow-up, each single research suggested that > 90% patients were satisfied with postoperative outcomes after LNF or LTF, and 91.78% (1674/1824) of patients in the LNF group and 91.33% (938/1027) in the LTF group reported excellent or good results after LARS. There was no statistically significant difference between the two groups (OR 0.96, 95% CI: 0.72-1.28, $P = 0.77$).

Objective evaluation

LES pressure: The change in LES pressure after LARS was investigated in 13 trials. The mean preoperative LES pressure was 3.1-12.8 mmHg in the LNF group, and about 2.3-13.6 mmHg in the LTF group, and increased significantly to 10.3-26 mmHg after LNF and 11-18 mmHg after LTF, respectively. Moreover, 360° total fundoplication could form a relatively stronger anti-reflux barrier than 270° partial funduplication because the amplitude of LES pressure increase was significantly higher after LNF than after LTF (OR 2.76, 95% CI: 1.57-3.95, $P < 0.05$).

Esophagitis: Five authors outlined the improvement of preoperative esophagitis after LARS by endoscopy. Esophagitis severity was graded according to the Savary-Miller classification in most studies. Remission of moderate-to-severe esophagitis was observed by endoscopic re-examination after anti-reflux procedures; there was a reduction in case number from 87/210 to 12/191 in the

Table 1 LNF and LTF in treatment of patients with gastroesophageal reflux disease

Data source	Design	PF (°)	Sample capacity (LNF/LTF)	Group or subg. depend on EM	Follow-up (mo)	Level of evidence
Mickevicius *et al*[22], 2008 Fibbe *et al*[23], 2001	RCT	200-270	76/77	No detail	12	1b Level A
Zornig *et al*[24], 2002 Strate *et al*[16], 2008	RCT	270	100/100	More subgroups	24	1b Level A
Chrysos *et al*[25], 2003	RCT	270	14/19	Both abnormal	12	2b Level B
Guérin *et al*[26], 2007	RCT	270	77/63	Normal-Nissen/Abnormal-Toupet	36	1b Level A
Laws *et al*[10], 1997	RCT	200	23/16	Both normal	27.2	2b Level B
Shaw *et al*[27], 2010	RCT	270	50/50	No detail	> 55	1b Level A
Booth *et al*[21], 2008	RCT	270	64/63	No detail	12	1b Level A
Coster *et al*[28], 1997	PCT	No detail	125/101	No detail	12	2b Level B
Granderath *et al*[29], 2007	PCT	270	28/28	Both normal	3	3b Level B
Wykypiel *et al*[30], 2008	PCT	300	20/20	Normal-Nissen/Abnormal-Toupet	6	3b Level B
Hunter *et al*[31], 1996	PCT	180-300	101/83	Both normal	3	3b Level B
Bessell *et al*[17], 2000	PCT	200-270	761/85	More subgroups	12	2b Level B
Radajewski *et al*[32], 2009	PCT	No detail	51/43	No detail	12	2b Level B
Kamolz *et al*[33], 2000 Kamolz *et al*[11], 2002	PCT	No detail	107/68	No detail	60	2b Level B
Sgromo *et al*[34], 2008	RT	No detail	150/116	No detail	72	2b Level B
Erenoğlu *et al*[35], 2003	RT	270	118/26	Normal-Nissen/Abnormal-Toupet	27.5	2b Level B
Herbella *et al*[20], 2007	RT	240	55/16	More subgroups	16	3b Level B
Wetscher *et al*[12], 1997	RT	No detail	17/32	Normal-Nissen/Abnormal-Toupet	15	3b Level B
Fernando *et al*[36], 2002	RT	270	163/43	No detail	19.7	2b Level B
Wykypiel *et al*[37], 2005	RT	300	77/132	Normal-Nissen/Abnormal-Toupet	52	2b Level B
McKernan *et al*[38], 1994	RT	180-200	14/14	No detail	> 3.4	3b Level B
Fein *et al*[45], 2008	RT	No detail	88/10	Normal-Nissen/Abnormal-Toupet	120	2b Level B
Patti *et al*[39], 2004	RT	240	216/141	More subgroups	> 23	2b Level B
Zügel *et al*[9], 2002	RT	270	40/122	No detail	19	2b Level B
Pessaux *et al*[40], 2000	RT	180	1078/392	No detail	24	2b Level B
Bell *et al*[41], 1996	RT	180	11/11	No detail	13	4 Level C
Lund *et al*[42], 1997	RT	270	16/46	Both abnormal	6	3b Level B
Farrell *et al*[43], 2000	RT	270	293/52	More subgroups	12	3b Level B
Heider *et al*[44], 2003	RT	270	15/4	Both abnormal	29.5	4 Level C

LNF: Laparoscopic Nissen fundoplication; LTF: Laparoscopic Toupet fundoplication; PF: Circumferential degrees of the partial wrap; Subg.: Subgroup; EM: Esophageal motility; RCT: Randomized controlled trial; PCT: Prospective controlled trial; RT: Retrospective trial.

Nissen group, and from 99/218 to 17/197 in the LTF group. There was no significant difference in remission rate between the two groups (6.28% *vs* 8.63%, OR 0.72, 95% CI: 0.59-1.33, $P = 0.42$).

Adverse results: Perioperative complications

Eleven studies reported the surgical complications in 1622 patients with LNF and 836 patients with LTF. Both techniques entailed a low (but definite) risk of surgical morbidity which occurred in 1.30%[26]-14.28%[25] of the population after Nissen fundoplication and in 2.46%[9]-15.63%[12] of the population after Toupet fundoplication. Lacerations of the gastric fundus and spleen, bleeding from the spleen or short gastric blood vessels and pneumothorax were the main disease categories. However, surgical mortality was also a natural sporadic event. Only two patients died: one on the 15th day after LNF due to secondary peritonitis from necrosis of the wrap[40], and the other died of esophageal perforation and mediastinitis[22]. The cumulative prevalence of perioperative complications after LNF and LTF was 4.01% and 5.14%, respectively, with no significant difference between the groups (4.01% *vs* 5.14%, OR 0.73, 95% CI: 0.47-1.12, $P = 0.15$).

Postoperative symptoms

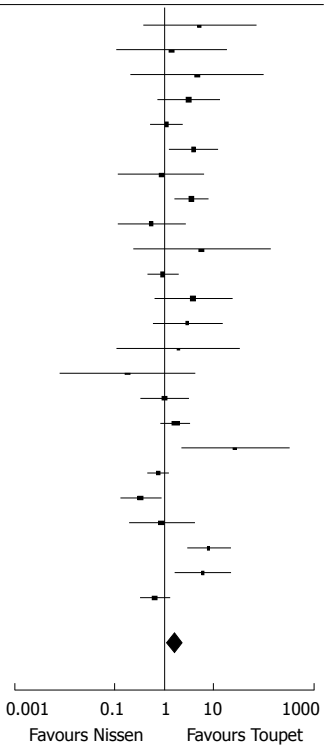
Dysphagia and need for bougie dilatation: Twenty-four studies assessed the outcome of dysphagia after LARS. Various questionnaires were used to define dysphagia severity, ranging from no symptoms to very severe episodes at the end of follow-up. The overall prevalence of dysphagia after LNF was significantly higher than after LTF (14.29% *vs* 11.00%, OR 1.68, 95% CI: 1.12-2.52, $P = 0.01$) (Figure 2A). A similar result was also obtained if only patients with moderate-to-severe dysphagia were included (12.30% *vs* 2.74%, OR 3.11, 95% CI: 1.94-5.00, $P < 0.01$). For patients with severe symptoms needing bougie dilatation, prevalence was still significantly higher after LNF than after LTF (7.91% *vs* 1.44%, OR 3.67, 95% CI: 1.90-7.09, $P < 0.01$). We thought it necessary to carry out subgroup analyses because preoperative EM was an important variable in the choice of surgical procedure and may have an effect on the analyses. Defining esophageal dysmotility was not consistent in the surgical literature. Most surgeons would agree that a distal esophageal amplitude > 40 mmHg and peristaltic contraction of the esophageal body of at least 70% indicate normal motility[18]. As described above, subgroup 1 analysis showed that the surgical procedure had no effect on the occurrence of

A

Study or subgroup	Nissen Events	Nissen Total	Toupet Events	Toupet Total	Weight (%)	Odds ratio M-H, random (95% CI)	Allocation concealment?
Shaw *et al*[27], 2010	3	7	1	8	1.9	5.25 (0.40-68.95)	Yes
Laws *et al*[10], 1997	2	23	1	16	2.0	1.43 (0.12-17.23)	Yes
Guérin *et al*[26], 2007	2	62	0	56	1.5	4.67 (0.22-99.38)	Yes
Mickevicius 3.0 *et al*[22], 2008	8	31	3	30	4.0	3.13 (0.74-13.20)	Yes
Patti *et al*[39], 2004	20	216	12	141	6.4	1.10 (0.52-2.32)	Yes
Booth *et al*[21], 2008	16	59	5	58	5.2	3.94 (1.34-11.63)	Yes
Chrysos *et al*[25], 2003	2	14	3	19	2.9	0.89 (0.13-6.18)	Yes
Strate *et al*[16], 2008	30	100	11	100	6.3	3.47 (1.62-7.40)	Yes
Mickevicius 1.5 *et al*[22], 2008	3	33	5	33	3.8	0.56 (0.12-2.56)	Yes
McKernan *et al*[38], 1994	2	14	0	14	1.4	5.80 (0.25-132.56)	No
Farrell *et al*[43], 2000	83	293	15	52	6.7	0.97 (0.51-1.87)	No
Wykypiel *et al*[30], 2008	6	20	2	20	3.3	3.86 (0.67-22.11)	No
Hunter *et al*[31], 1996	7	101	2	83	3.6	3.02 (0.61-14.93)	No
Wetscher *et al*[12], 1997	1	17	1	32	1.6	1.94 (0.11-33.05)	No
Granderath *et al*[29], 2007	0	28	2	28	1.4	0.19 (0.01-4.05)	No
Erenoğlu *et al*[35], 2003	23	118	5	26	5.2	1.02 (0.35-2.98)	No
Sgromo *et al*[34], 2008	48	99	22	62	6.7	1.71 (0.89-3.29)	No
Bell *et al*[41], 1996	10	11	3	11	2.1	26.67 (2.31-308.00)	No
Pessaux *et al*[40], 2000	57	1078	27	392	7.3	0.75 (0.47-1.21)	No
Fernando *et al*[36], 2002	17	113	10	29	5.7	0.34 (0.13-0.85)	No
Fein *et al*[45], 2008	23	74	3	9	3.9	0.90 (0.21-3.93)	No
Wykypiel *et al*[37], 2005	21	77	6	132	5.6	7.88 (3.01-20.57)	No
Lund *et al*[42], 1997	10	16	10	46	4.7	6.00 (1.75-20.55)	No
Bessell *et al*[17], 2000	87	761	14	85	6.9	0.65 (0.35-1.21)	No
Total (95% CI)		3365		1482	100	1.68 (1.12-2.52)	
Total events	481		163				

Heterogeneity: Tau^2 = 0.53; χ^2 = 65.84, *df* = 23 (*P* < 0.00001); I^2 = 65%

Test for overall effect: *Z* = 2.50 (*P* = 0.01)



B

Study or subgroup	Nissen Events	Nissen Total	Toupet Events	Toupet Total	Weight (%)	Odds ratio M-H, random (95% CI)	Allocation concealment?
2.2.1 Motility normal (both)							
Strate *et al*[16], 2008	16	50	3	50	16.7	7.37 (1.99-27.32)	Yes
Laws *et al*[10], 1997	2	23	1	16	7.8	1.43 (0.12-17.23)	Yes
Bessell *et al*[17], 2000	58	558	5	21	19.9	0.37 (0.13-1.05)	No
Hunter *et al*[31], 1996	7	101	2	83	13.7	3.02 (0.61-14.93)	No
Farrell *et al*[43], 2000	75	267	9	31	22.7	0.95 (0.42-2.17)	No
Fernando *et al*[36], 2002	14	88	4	7	13.7	0.14 (0.03-0.70)	No
Granderath *et al*[29], 2007	0	28	2	28	5.6	0.19 (0.01-4.05)	No
Subtotal (95% CI)		1115		236	100	0.93 (0.32-2.64)	
Total events	172		26				

Heterogeneity: Tau^2 = 1.31; χ^2 = 21.66, *df* = 6 (*P* = 0.001); I^2 = 72%

Test for overall effect: *Z* = 0.14 (*P* = 0.89)

2.2.2 Motility abnormal (both)							
Patti *et al*[39], 2004	9	55	12	141	19.3	2.10 (0.83-5.32)	Yes
Strate *et al*[16], 2008	14	50	8	50	18.7	2.04 (0.77-5.42)	Yes
Chrysos *et al*[25], 2003	2	14	3	19	9.9	0.89 (0.13-6.18)	Yes
Farrell *et al*[43], 2000	8	26	6	21	15.6	1.11 (0.31-3.92)	No
Bessell *et al*[17], 2000	29	203	9	64	20.7	1.02 (0.45-2.28)	No
Lund *et al*[42], 1997	10	16	10	46	15.9	6.00 (1.75-20.55)	No
Subtotal (95% CI)		364		341	100	1.75 (1.04-2.94)	
Total events	72		48				

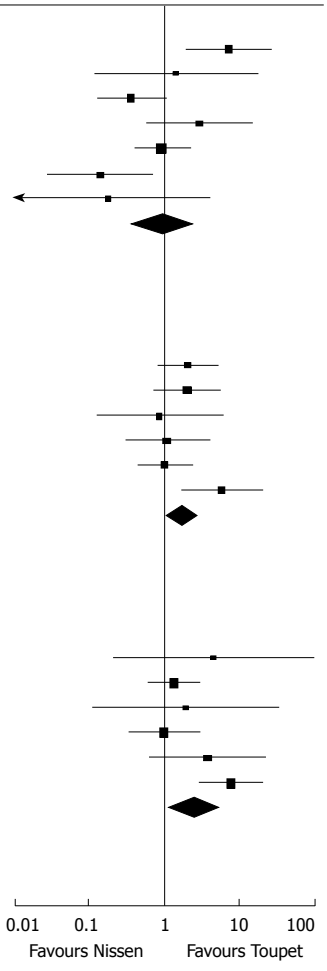
Heterogeneity: Tau^2 = 0.11; χ^2 = 6.78, *df* = 5 (*P* = 0.24); I^2 = 26%

Test for overall effect: *Z* = 2.11 (*P* = 0.04)

2.2.3 Motility normal (Nissen) *vs* motility abnormal (Toupet)							
Guérin *et al*[26], 2007	2	62	0	56	6.4	4.67 (0.22-99.38)	Yes
Patti *et al*[39], 2004	18	161	12	141	26.5	1.35 (0.63-2.92)	Yes
Wetscher *et al*[12], 1997	1	17	1	32	7.2	1.94 (0.11-33.05)	No
Erenoğlu *et al*[35], 2003	23	118	5	26	22.0	1.02 (0.35-2.98)	No
Wykypiel *et al*[30], 2008	6	20	2	20	14.1	3.86 (0.67-22.11)	No
Wykypiel *et al*[37], 2005	21	77	6	132	23.7	7.88 (3.01-20.57)	No
Subtotal (95% CI)		455		407	100	2.47 (1.08-5.68)	
Total events	71		26				

Heterogeneity: Tau^2 = 0.51; χ^2 = 10.94, *df* = 5 (*P* = 0.05); I^2 = 54%

Test for overall effect: *Z* = 2.14 (*P* = 0.03)



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Study or subgroup	Nissen Events	Nissen Total	Toupet Events	Toupet Total	Weight (%)	Odds ratio M-H, random (95% CI)	Allocation concealment?
3.3.1 Postoperative gas bloating							
Chrysos *et al*[25], 2003	3	14	3	19	6.7	1.45 (0.25-8.58)	Yes
Guérin *et al*[26], 2007	1	62	0	56	3.1	2.76 (0.11-69.04)	Yes
Booth *et al*[21], 2008	11	59	6	58	9.7	1.99 (0.68-5.79)	Yes
Wykypiel *et al*[37], 2005	18	77	6	132	10.1	6.41 (2.42-16.97)	No
Sgromo *et al*[34], 2008	33	99	23	62	11.5	0.85 (0.44-1.65)	No
Wykypiel *et al*[30], 2008	6	20	0	20	3.6	18.38 (0.96-352.57)	No
Granderath *et al*[29], 2007	17	28	7	28	9.3	4.64 (1.48-14.54)	No
Fernando *et al*[36], 2002	60	113	18	29	10.8	0.69 (0.30-1.60)	No
Bell *et al*[41], 1996	9	11	3	11	5.8	12.00 (1.58-91.08)	No
Fein *et al*[45], 2008	63	74	5	9	7.9	4.58 (1.06-19.78)	No
Coster *et al*[28], 1997	70	82	38	62	10.9	3.68 (1.66-8.18)	No
Radajewski *et al*[32], 2009	36	51	29	43	10.6	1.16 (0.48-2.79)	No
Subtotal (95% CI)		690		529	100	2.42 (1.37-4.26)	
Total events	327		138				
Heterogeneity: Tau^2 = 0.55; χ^2 = 30.48, *df* = 11 (*P* = 0.001); I^2 = 64%							
Test for overall effect: *Z* = 3.06 (*P* = 0.002)							
3.3.2 Postoperative inability to belch							
Guérin *et al*[26], 2007	0	62	2	56	4.9	0.17 (0.01-3.71)	Yes
Booth *et al*[21], 2008	34	59	24	58	16.0	1.93 (0.92-4.02)	Yes
Wykypiel *et al*[30], 2008	6	20	0	20	5.1	18.38 (0.96-352.57)	No
Sgromo *et al*[34], 2008	74	99	55	62	14.9	0.38 (0.15-0.93)	No
Granderath *et al*[29], 2007	26	28	7	28	10.1	39.00 (7.32-207.87)	No
Fernando *et al*[36], 2002	22	113	5	29	13.8	1.16 (0.40-3.38)	No
Wykypiel *et al*[37], 2005	26	77	9	132	15.4	6.97 (3.05-15.90)	No
Coster *et al*[28], 1997	12	82	0	62	5.4	22.16 (1.29-382.06)	No
Radajewski *et al*[32], 2009	20	51	7	43	14.4	3.32 (1.24-8.89)	No
Subtotal (95% CI)		591		490	100	3.02 (1.14-7.95)	
Total events	220		109				
Heterogeneity: Tau^2 = 1.54; χ^2 = 42.66, *df* = 8 (*P* < 0.00001); I^2 = 81%							
Test for overall effect: *Z* = 2.23 (*P* = 0.03)							

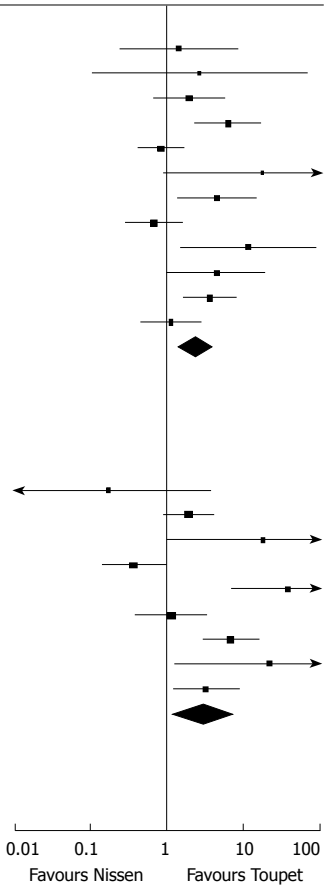


Figure 2 Pooled analysis using the Mantel-Haenszel method and a random-effect model. A: Overall rates of dysphagia after laparoscopic Nissen fundoplication (LNF) and laparoscopic Toupet fundoplication (LTF); B: Subgroup dysphagia depending on preoperative esophageal motility after LNF and LTF; C: Postoperative gas bloating and inability to belch after LNF and LTF.

postoperative dysphagia if preoperative EM was normal (*P* = 0.89). However, in subgroup 2, patients with abnormal EP, the prevalence of dysphagia after LNF was still more common than after LTF (*P* = 0.04). In subgroup 3, even though the surgical choice was in accordance with the so-called "tailored procedure", LNF also led to a significantly higher risk of developing postoperative dysphgia than LTF (*P* = 0.03) (Figure 2B).

Gas-bloat syndrome or gas-related symptoms: Gas-related symptoms ("gas-bloat syndrome") were described in 12 studies involving > 1200 patients. Gas-bloat syndrome included gas bloating, inability to belch, flatulence, postprandial fullness, and epigastric pain. The main objective evaluation of gas-related symptoms was done using a verbal rating scale using the items mentioned above. Overall, 47.39% (327/690) of patients after LNF and 26.09% (138/529) of patients after LTF suffered from gas bloating, whereas 37.22% (220/591) after LNF and 22.24% (109/490) after LTF were unable to belch. Gas bloating and inability to belch were more common in the LNF group and a significant difference between LNF and LTF was also further confirmed to reach a significant level (Figure 2C).

Recurrence and re-operation

Heartburn and reflux recurrence: Eleven studies investigated the incidence of postoperative heartburn (considered to be the cardinal symptom of GERD recurrence). Heartburn occurred in 6.45%[26]-60.29%[43] of the population after LNF, and in 5.26%[25]-55.10%[43] after LTF. No statistically significant difference was found between LNF and LTF concerning the cumulative prevalence of heartburn (32.97%, 331/1004 *vs* 31.09%, 157/505, OR 0.83, 95% CI: 0.52-1.33, *P* = 0.45, respectively).

Nine studies reported the postoperative recurrence of GERD. However, the standard of GERD recurrence was not uniform because some were judged according to symptoms such as reflux, chest pain, and heartburn, whereas others were based on the endoscopic characteristics and 24-h gastric monitoring of pH (e.g. persistent esophagitis, DeMeester score[46]). In our analysis, the cumulative prevalence of reflux recurrence was slightly higher after LTF than after LNF, but this difference was not significant (6.50%, 64/985 *vs* 9.42%, 84/892, OR 0.73, 95% CI: 0.35-1.53, *P* = 0.40).

Re-operation: Re-operation prevalence was described in 15 trials involving > 3800 patients. The main causes

of re-operation were persistent dysphagia, severe reflux symptoms, hiatus hernia recurrence, and other severe treatment failures. Although the re-operation rate after LNF was slightly higher than after Toupet fundoplication, 4.40% (115/2616) *vs* 3.68% (47/1276), the difference was not significant (OR 1.29, 95% CI: 0.73-2.29, $P = 0.38$). The morbidity associated with re-operation after LNF for GERD ranged from 1.56%[21] to 27.27%[41], and in the LTF group the rates varied from 1.00%[28] to 17.5%[22].

DISCUSSION

Lower morbidity and mortality, shorter hospitalization and faster convalescence made LARS so attractive to patients with GERD. Since Nissen procedure was first reported a decade ago, it has been adopted as a tremendously successful procedure for reflux control, and it is therefore more often performed than partial fundoplication. However, numerous recent researches witnessed a strong debate between Nissen and Toupet fundoplications[47], shifting the attention to postoperative failures due to mechanical problems (e.g. dysphagia), rather than worries about the recurrence of disease.

Our analysis is a comprehensive and detailed systematic literature review of 32 articles reporting the surgical outcomes of 6236 patients with GERD after LNF and LTF. It demonstrated that the latter is advantageous over the former, showing similar outcomes of satisfaction rate, endoscopic improvement, perioperative complication occurrence, reflux recurrence and re-operation, but a substantially reduced prevalence of postoperative symptoms (e.g. dysphagia, gas-bloat syndrome).

Patient satisfaction is a reasonable and accurate index for assessing the efficacy of surgical treatment for GERD[11,47]. In our meta-analysis, > 90% of the study populations reported excellent or good results in the LNF and LTF groups. Bearing in mind that the subjective description of patients was not always in accordance with objective findings[48], we further analyzed the alteration of LES pressure and remission of esophagitis after construction of a mechanical anti-reflux barrier. Elevating the resting pressure of the LES played a crucial role in controlling reflux symptoms, blocking the natural history of Barrett's esophagus and reducing the risk of malignancy. We also found that LNF and LTF could increase the LES pressure and improve preoperative esophagitis. Even though the amplitude of elevation of LES pressure was significantly lower after LTF than after LNF, it seemed that it remained sufficiently powerful to resist the reflux of gastric contents. These subjective assessments and objective evaluations supported the point of view that LTF can control reflux symptoms for GERD.

The prevalence of perioperative complications showed no significant difference between groups, indicating that neither procedure was more technically difficult or more demanding of surgical skills. However, the prevalence of postoperative dysphagia and gas-related symptoms was much higher after LNF than after LTF. Even though the mechanism of gas-related symptoms is unclear[29], these results suggest that wrap type and alteration in LES pressure were the underlying causes.

Dysphagia was still the complication of greatest concern and presented in three scenarios: acute total dysphagia immediately after surgery; mild dysphagia within the first postoperative 6-8 wk; and persistent chronic dysphagia after the postoperative 6-8 wk[18,49-51]. Some authors thought that dysphagia converges and the difference normalizes within 12 mo[52-55]. This study analyzed the prevalence of chronic dysphagia at the end of follow-up, and the mean duration of follow-up of most included studies was > 12 mo. A subgroup analysis was also executed to incorporate the variable of EM. It seemed that in normal motility patients (subgroup 1), extent of anti-reflux barrier did not affect the possibility of dysphagia. But in patients with abnormal motility (subgroup 2), the higher dysphagia rate after LNF supported that the tailored approach should be preserved. Even using the tailored approach (subgroup 3), the prevalence of dysphagia after LTF was still significantly lower than after LNF. These results indicated that, irrespective of EM status, LTF would be a safer choice in reducing this complication.

Concerns regarding GERD recurrence made surgeons select the Nissen technique rather than the Toupet technique for a long time. The similar prevalence of reflux recurrence between LNF and LTF groups in our analyses appeared to confirm the effectiveness of the Toupet procedure from another viewpoint. However, the value of this index may be slightly inferior. A DeMeester score > 14.7 was a generally accepted criterion, but the standards of GERD recurrence of selected researches were not uniform. Some were judged based on symptoms, some on endoscopic manometry. Furthermore, even though specific pH changes in the gastric environment were related to symptoms to some extent, inconsistency of poor correlation between postoperative reflux symptoms and reflux abnormal DeMeester scores were noted in many instances[27,45,48].

For re-operation, we did not divide subgroups according to causes such as recurrence of reflux or complications. The reason was that they were a miserable experience for the patients and also indicated the failure of primary surgery. The re-operation prevalence between the two groups was comparable. A remarkable phenomenon discovered in some studies[16,23,24] was that a large proportion of patients needing re-operation after LNF possessed a intact wrap during surgery with herniation of the wraps into the mediastinum. Nevertheless, it was rarely detected and presented after LTF. The technical issue of a suture between the posterior wall of the wrap and the two crura of the diaphragm, or a suture between the upper portion of the wrap and the anterior edge of the hiatus, has been proposed to maintain the wrap in an adequate abdominal position and to avoid its rotation and migration.

In conclusion, the results of the present meta-analysis suggested that LTF might be the current procedure of choice to treat GERD. It should be advocated as a more physiologic alternative for Nissen repair, allowing a reduced morbidity rate with similar efficacy in reflux control

and recurrence. The surgical patterns might be a prior factor to preoperative esophageal motility in affecting the postoperative dysphagia after LARS. More multicenter, randomized controlled trials including objective outcome assessment are required to further confirm the value of LNF and LTF.

COMMENTS

Background

The laparoscopic Nissen fundoplication and the laparoscopic Toupet fundoplication are the two major anti-reflux surgical procedures for gastroesophageal reflux disease (GERD). There has been much debate about the superiority of these two techniques.

Research frontiers

A decade ago, Nissen procedure was first reported, then adopted as a tremendous successful procedure for reflux control, and was therefore more often performed than posterior partial fundoplication. However, numerous recent researches witnessed a strong debate about Nissen and Toupet fundoplication, shifting the attention to postoperative failures due to mechanical problems (e.g. dysphagia), rather than worries about the recurrence of disease.

Innovations and breakthroughs

To the best of the authors' knowledge, the present study was the most comprehensive and detailed systematic literature review to demonstrate the optimal surgical procedure for GERD.

Applications

The research showed that laparoscopic Toupet fundoplication might be the current procedure of choice to treat GERD. It should be advocated as a more physiologic alternative for Nissen repair, allowing a reduced morbidity rate with similar efficacy in reflux control and recurrence.

Peer review

This article is important and interesting.

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BRIEF ARTICLE

Simultaneous detection of different serum pepsinogens and its primary application

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Abstract

AIM: To develop the simple, rapid and sensitive dual-label time-resolved fluoroimmunoassay for pepsinogens in human serum.

METHODS: Based on two-site sandwich protocol, monoclonal antibodies (McAbs) against pepsinogen Ⅰ (PG Ⅰ) and PG Ⅱ were co-coated in 96 microtitration wells, and tracer McAbs against PG Ⅰ and PG Ⅱ were labeled with europium (Eu) and samarium (Sm) chelate, respectively. Diluted serum samples of Eu^{3+}- and Sm^{3+}-McAbs were added into microtitration wells simultaneously. After washing, fluorescence of bound Sm^{3+} and Eu^{3+} tracers was detected.

RESULTS: The detection limit was 0.2 μg/L for PG Ⅰ and 0.05 μg/L for PG Ⅱ. The assay range was 5.0-320.0 μg/L for PG Ⅰ and 1.0-55.0 μg/L for PG Ⅱ. The average recovery rate was 102.7% for PG Ⅰ and 98.8% for PG Ⅱ. Sera from healthy controls and patients with gastric disease were analyzed. The PG detected by dual-label assay was in good agreement with that detected by single-label assay or by enzyme-linked immunosorbent assay.

CONCLUSION: Dual-label assay can provide high-throughput serological screening for gastric diseases.

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Key words: Serum pepsinogen; Simultaneous detection; Time-resolved fluoroimmunoassay

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INTRODUCTION

There is considerable interest in developing methods to simultaneously quantify two or more analyses of one sample in a single assay[1,2], which is advantageous to the confidence level of results, especially in cases when the ratio of compounds gives important information[3,4]. Dual-label has potential applications in various fields such as microbiology, molecular biology, drug analysis, and clinical research. Fluorescence immunoassay, like other immunoassays involving non-isotopic labeling, has been well accepted as a stable, inexpensive, rapid, and sensitive

method. However, conventional fluorescent labeling has a limited success in assay of multiple analytes because of its high background, short decay time and broad spectrum, which make it difficult to be distinguished between its emission bands[5,6]. Up to now, fluorescent lanthanide is a favorable choice owing to its narrow emission peak at different wavelengths. Its lifetime ranges 50-1000 μs (over four decades longer than the average background duration) depending on the temperature and the solvent presented[7]. These features can be utilized for optimization of the measurement conditions to get the maximal sensitivity and to minimize the signal spillover. The europium ion (Eu^{3+}), is the lanthanide mainly used in time-resolved fluoroimmunoassay (TRFIA)[8]. Eu^{3+} and terbium ion (Tb^{3+}) form the most efficient fluorescent chelates, but Tb^{3+} requires fluorinated aliphatic β-diketone for simultaneous detection[9], rather than β-naphthoyltrifluoroacetone (β-NTA) used in the enhancement solution optimized for Eu^{3+} detection. β-NTA is also applicable to samarium ion (Sm^{3+}) excitation, which has thus been suggested that Sm^{3+} can be used as a counterpart to Eu^{3+} with the same enhancement formulation (enhancement solution or DELFIA inducer) in a dual-label system[10].

Human pepsinogens originating from gastric mucosa can be classified into two immunochemically distinct groups: pepsinogen I (PG I) and PG Ⅱ[11], which are mostly secreted into the gastric lumen and nearly 1% of them are leaked into the blood circulation. Serum PG levels reflect the morphological and functional status of gastric mucosa. Human pepsinogens have a diagnostic value for various gastroduodenal disorders, especially for peptic ulcer and atrophic gastritis, which have been widely discussed[12,13]. The PG I /PG Ⅱ ratio can provide even better information on the extent of chronic gastritis than gastric intubation[14].

Since PG I and PG Ⅱ serve as useful predictors in early diagnosis of gastric cancer and in mass screening of populations at a high risk of gastric cancer[15,16], a reliable and sensitive method is needed to detect PG I and PG Ⅱ in human sera. In our previous study[17], a fast and highly sensitive TRFIA was developed to measure serum PG I and PG Ⅱ. The present study was to evaluate the dual-label TRFIA for simultaneous detection of PG I and PG Ⅱ in human serum.

MATERIALS AND METHODS

Chemicals and instruments

Diethylenetriaminepentaacetate (DTPA), bovine serum albumin (BSA), Tris and Triton X-100 were purchased from Sigma (St. Louis, MO, USA). PD-10 column and sepharoseCL-6B column were from the Pharmacia Company (Chalfont St Giles, UK). Q2 anion exchange chromatography, DEAE-52 chromatography, and gel filtration HPLC were purchased from Bio-Rad Company (Hercules, USA). Pure water was produced by Barnstead Equipment (Dubuque, Iowa, USA). Ninety six -well polystyrene microtitre plates were obtained from Nunc International (Roskilde, Denmark). Eu-labeling reagent 1244-302 and Sm-labeling reagent 1244-303, both including N'-[*p*-isothiocyanatobenzyl]-diethylenetriamine-N^1, N^2, N^3, N^4-tetraacetic acid, were purchased from Perkin-Elmer (Waltham, Massachusetts, USA). β-NTA was synthesized in our laboratory. Two counterparts of monoclonal antibodies (McAbs) to human PG I and PG Ⅱ respectively for capture and detection were obtained from Chinese Institute of Cancer with a purity of over 95% (Beijing, China). Enzyme-linked immunosorbent assay (ELISA) kits for detection of PG I and PG Ⅱ were from Biohit Plc(Helsinki, Finland). AutoDELFIA1235, from Perkin-Elmer (Waltham, Massachusetts, USA), was used to measure Eu^{3+} and Sm^{3+} fluorescence in microtiter wells. All other reagents used were of analytical grade.

Reagent solutions

Labeling buffer contained 50 mmol/L Na_2CO_3-$NaHCO_3$ (pH 8.5), and 0.155 mol/L NaCl. Elution buffer contained 50 mmol/L Tris-HC1 (pH 7.8), 0.9% NaC1, and 0.05% NaN_3. Assay buffer contained 50 mmol/L Tris-HCl (pH 7.8), 0.9% NaC1, 0.2% BSA, 0.05% NaN_3, 20 μmol/L DTPA, and 0.1% Tween-20. Washing solution was a Tris-HCl buffered saline solution (pH 7.8) containing 0.9% NaCl, 0.2% Tween-20, and 0.05% NaN_3. Enhancement solution was a 0.1 mol/L acetate-phthalate buffer (pH 3.2) containing 0.1% triton X-100, 15 μmol /L βNTA, and 50 μmol/L tri-*n*-octylphosphine oxide.

Serum samples

Serum samples, collected from healthy volunteers who had no upper abdominal complaints and evidence of gastroduodenal disorder and liver diseases, were stored at -20℃. Blood samples were collected from patients at endoscopic and histological examinations. This study was conducted with the approval of the Ethics Committee of Jiangyuan Hospital Affiliated to Jiangsu Institute of Nuclear Medicine.

Immobilization of McAbs

Five micrograms of the capture McAbs to PG I and PG Ⅱ in 200 μL of 50 mmol of Na_2CO_3-$NaHCO_3$ buffer (pH 9.6) was co-immobilized in each well and incubated overnight at room temperature. After washing, 200 μL of 1 g/L BSA in 50 mmol of Na_2CO_3-$NaHCO_3$ buffer (pH 9.6) was added to block the coated surface for 2 h. After the blocking solution was removed, the plates were dried in a high vacuum, and then stored at -20℃ in a sealed plastic bag with desiccant.

Labeling of McAbs with Eu^{3+}- and Sm^{3+}-chelates

McAbs to human PG I (PG I McAbs) and PG Ⅱ (PG Ⅱ McAbs) were labeled with Sm^{3+}- and Eu^{3+}-chelates, respectively. The buffer for McAbs was replaced with the labeling buffer. Five hundred micrograms of PG I McAbs was gently mixed in 200 μL of labeling buffer with 250 μg of Sm^{3+}-chelates in 100 μL of the same buffer. After an 18-h incubation with continuous gentle shaking at room

temperature, free Sm^{3+}-chelates and aggregated McAbs were separated from Sm^{3+}-McAbs conjugates using a 1 cm × 40 cm column packed with sepharose CL-6B (lower 20 cm), eluted with a descending elution buffer, and collected with 1.0 mL per fraction. The concentration of Sm^{3+}-conjugates in collected fraction was measured with fluorescence, and diluted with an enhancement solution (1:1000). The fluorescence in microtitration wells (200 μL per well) was detected by comparing the signal of samples to that of stock standards diluted at 1:100 in an enhancement solution. The fractions from the first peak with the highest Sm^{3+} count were pooled and characterized. PG Ⅱ McAbs were labeled with Eu. The labeled McAbs were rapidly lyophilized under high vacuum after dilution with an elution buffer containing 0.2% BSA as a stabilizer, and stored at -20℃. No loss of immunoreactivity was observed during a 6-mo storage period.

Purification of PG and calibrators

Surgically resected stomach tissues were free from the invaded part. PG Ⅰ and PG Ⅱ were purified by DEAE-52 chromatography, gel filtration HPLC, and Q2 anion exchange chromatography, as previously described[18]. The purity of PG Ⅰ was over 98% and that of PG Ⅱ was over 95.0%. Calibrators were prepared by diluting them with the assay buffer containing 0, 5, 10, 50, 100, 300 μg/L of highly purified PG Ⅰ and 0, 5, 10, 20, 30, 50 μg/L of highly purified PG Ⅱ, respectively.

Assay procedure

Dual-label TRFIA was performed to detect PG Ⅰ and PG Ⅱ simultaneously in serum with a one-step "sandwich-type" protocol. In brief, 25 μL of calibrators (samples) and 200 μL of 50-fold diluted Eu^{3+} and Sm^{3+} tracer McAbs solution in assay buffer were pipetted into the coated microtitration wells. The plates were incubated with continuous shaking for 2 h at 25℃. After washed 6 times, 200 μL of enhancement solution was added into each well. The plates were shaken for 5 min before fluorescence reading. All procedures were automatically performed by $_{auto}$DELFIA$_{1235}$ with the software designed in our laboratory. Calibration curve was plotted and concentrations in unknown samples were measured using Multicalc software program, where a spline algorithm on logarithmically transformed data was employed. ELISA was performed with a kit following its instructions.

Statistical analysis

Data about PG Ⅰ or PG Ⅱ were expressed as mean ± SD. The limit of detection was defined by the concentration of PG Ⅰ or PG Ⅱ corresponding to the fluorescence of the zero calibrators plus two SD. The average intra- or inter-assay coefficient of variation (CV) was calculated for the precision of the assay. The recovery rate was evaluated by comparing the measured and theoretical values. Regression analysis was used to display the linearity and correlations. Differences in patients with gastric disease and healthy controls were analyzed using paired *t*-test. $P < 0.05$ was considered statistically significant. Analysis of data was performed using SPSS 13.0 (Chicago, IL, USA).

Table 1 Precision of dual-label assay for PG Ⅰ and PG Ⅱ in serum of controls[1]

	PG Ⅰ (μg/L)		PG Ⅱ (μg/L)	
	mean ± SD	CV (%)	mean ± SD	CV (%)
Serum pool 1				
Within-run (n = 10)	43.2 ± 1.36	3.2	5.23 ± 4.62	4.8
Between-run (n = 6)	42.8 ± 2.28	5.1	5.65 ± 6.45	6.7
Serum pool 2				
Within-run (n = 10)	105.0 ± 1.88	2.3	11.7 ± 3.66	3.9
Between-run (n = 6)	103.8 ± 2.73	3.6	10.5 ± 4.55	5.1
Serum pool 3				
Within-run (n = 10)	198.2 ± 5.31	6.3	22.0 ± 3.88	4.6
Between-run (n = 6)	186.7 ± 4.35	5.4	21.2 ± 5.71	8.3

[1]Served as controls. PG: Pepsinogen; CV: Coefficient of variation.

RESULTS

Kinetics, detection limits and precision

The calibrators covered a range of 10-300 μg/L of PG Ⅰ and 2-50 μg/L of PG Ⅱ. The serum samples from healthy controls and patients with chronic atrophic gastritis and peptic ulcer were incubated for different periods of time (60, 90, 120, 150 min) at different temperatures (25℃, 37℃). Both calibrators and serum samples reached a plateau value around 120 min at 25℃ and around 60 min at 37℃, respectively. In this study, the incubation time was 120 min and the temperature was 25℃ for the assay on $_{auto}$DELFIA$_{1235}$.

The calibration curves of PG Ⅰ and PG Ⅱ were linear over the concentration. The equation was y = 46.4x + 383.2 for the calibration curve of PG Ⅰ and y = 2198.2x + 5189.1 for the calibration curve of PG Ⅱ, where y indicates the response counts (cps), x indicates the concentration (μg/L). With 25 μL of serum samples, the measurement range of PG Ⅰ, ED_{20}, ED_{50}, and ED_{80} was 5.0-320.0, 19.87 ± 4.3, 64.32 ± 6.2, and 176.0 ± 12.9 μg/L, respectively, and the measurement range of PG Ⅱ, ED_{20}, ED_{50}, and ED_{80} was 1.0-55.0, 3.546 ± 2.2, 9.746 ± 4.7, and 23.79 ± 6.3 μg/L, respectively.

The limit of detection was 0.2 μg/L for PG Ⅰ and 0.05 μg/L for PG Ⅱ. The average intra-assay CV of the calibrators was 4.6% for PG Ⅰ and 5.3% for PG Ⅱ. The intra- and inter-assay CV of serum-based controls was summarized in Table 1. The results showed that the assay had a good precision not only for the calibrators but also for the clinical samples.

The cross-reactivity between anti-PG Ⅰ antibody to PG Ⅱ and anti-PG Ⅱ antibody to PG Ⅰ was detected. No interference between them was found. The result showed that the specificity of the assay was good.

Analysis of samples

The correlations between dual-label and single-label assay are shown in Figure 1. The correlation ratio between

Table 2 Serum PG level and PG Ⅰ/PG Ⅱ ratio in healthy controls and patients with duodenal ulcer, gastric ulcer, atrophic gastritis, superficial gastritis and gastric cancer (mean ± SD)

Diagnosis	*n*	Age (yr)	PG Ⅰ (μg/L)	PG Ⅱ (μg/L)	PG Ⅰ/PG Ⅱ ratio
Healthy controls	500	41.5 ± 18.8	150.3 ± 45.1	10.4 ± 8.4	14.5 ± 4.3
Patients with duodenal ulcer	112	43 ± 14.2	252.9 ± 84.5[d]	18.4 ± 16.0[d]	13.7 ± 8.0
Patients with gastric ulcer	44	37 ± 11.2	221.2 ± 91.7[d]	15.6 ± 12.4[b]	14.2 ± 7.1
Patients with atrophic gastritis	21	48 ± 9.7	89.5 ± 51.2[d]	12.9 ± 9.39	6.9 ± 6.2[b]
Patients with superficial gastritis	76	35 ± 7.4	175.3 ± 45.8[a]	12.7 ± 10.1	13.8 ± 5.3
Patients with gastric cancer	126	55 ± 12.2	157.1 ± 81.9	15.6 ± 14.4[b]	13.4 ± 7.8

[a]$P < 0.05$, [b]$P < 0.01$, [d]$P < 0.001$ *vs* healthy controls.

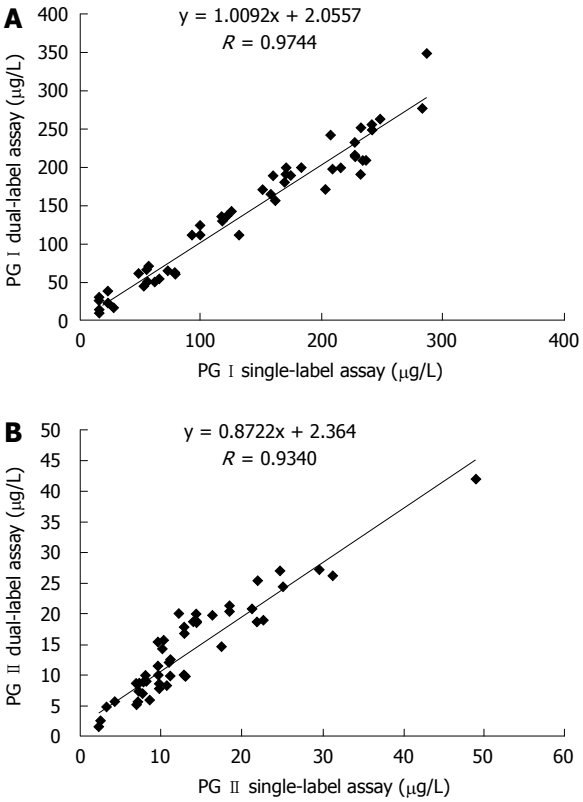


Figure 1 Correlation between single-label and dual-label assay of pepsinogen (PG) Ⅰ (A) and PG Ⅱ (B) in human sera (*n* = 50).

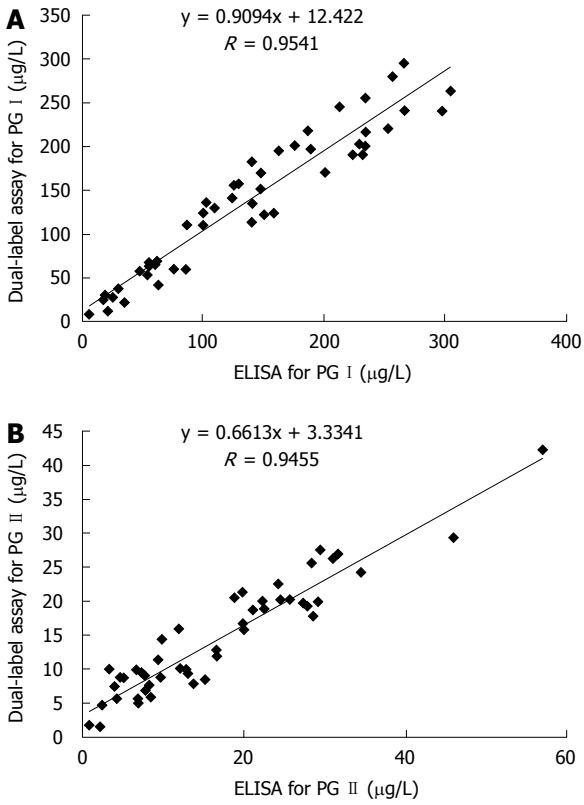


Figure 2 Correlation between enzyme-linked immunosorbent assay (ELISA) and dual-label assay of PG Ⅰ (A) and PG Ⅱ (B) in human sera (*n* = 50).

single-label and dual-label assay for PG Ⅰ and PG Ⅱ was 0.9744 and 0.9340, respectively. The correlations between dual-label assay and ELISA for PG Ⅰ and PG Ⅱ are shown in Figure 2. The correlation ratio between ELISA and dual-label for PG Ⅰ and PG Ⅱ was 0.9541 and 0.94550, respectively. The results indicate that the dual-label assay is in agreement with ELISA or with single-label assay.

The serum samples were analyzed by dual-label assay (Table 2). The normal range of serum PG Ⅰ in healthy controls was 60.1-240.3 μg/L. The range of serum PG Ⅱ was lower than 27.2 μg/L. The cut-off point was 5.9 for the PG Ⅰ/PG Ⅱ ratio.

The serum PG level was lower or higher than its normal range in dyspeptic patients including those with gastric cancer. The distributions of PG Ⅰ and PG Ⅱ value and PG Ⅰ/PG Ⅱ ratio in patients with gastric cancer and in those with duodenal ulcer are shown in Table 3. The difference in PG Ⅰ value was relatively small. The serum PG Ⅰ level was remarkably higher in peptic ulcer patients, especially in those with active duodenal ulcer than in healthy controls. The increased PG Ⅰ level would be a high risk factor for duodenal ulcer, and a remarkably low serum PG Ⅰ level could exclude the diagnosis of peptic ulcer[19].

DISCUSSION

Eu^{3+} chelate is the most commonly used label in time-resolved fluorometry-based analysis because of its higher fluorescence yield and lower background than other lanthanide complexes. Tb^{3+} chelate usually has a longer decay time and a higher fluorescence yield than Sm^{3+} chelate, and their fluorescence is less sensitive to aqueous quench-

Table 3 PG Ⅰ and PG Ⅱ values and PG Ⅰ/PG Ⅱ ratio in patients with gastric carcinoma and duodenal ulcer *n* (%)

	n	PG Ⅰ (μg/L)			PG Ⅱ (μg/L)		PG Ⅰ/PG Ⅱ ratio	
		< 60	60-240	> 240	≤ 27	> 27	≤ 6	> 6
Patients with gastric cancer	126	17 (13.5)	88 (69.8)	21 (16.7)	109 (86.5)	17 (13.5)	25 (19.8)	101 (80.2)
Patients with duodenal ulcer	112	0	69 (61.6)	43 (38.4)	100 (89.2)	12 (10.8)	3 (2.7)	109 (97.3)

ing. However, the relatively shorter emission wavelength of Tb^{3+} chelate (545 nm) makes it more prone to interference (e.g. phosphorescence) derived from plastic or glass materials. Additionally, it is required to use an aliphatic β-diketone to enhance the fluorescence of Tb^{3+} in immunoassay for DELFIA-type of multiple analytes[9]. Considering these factors, we selected Eu^{3+} and Sm^{3+} as labels in the present study.

As the Sm photoluminescence yield is lower than that of Eu, Sm^{3+} is usually used as a tracer in assays not requiring a great sensitivity. The detection limit for PG Ⅰ in dual-label assay is 0.2 μg/L, whereas that of single-label assay is 0.05 μg/L[17]. The sensitivity and precision for PG Ⅰ can be improved significantly by increasing Sm^{3+} label yield. The labeling reaction between PG Ⅰ McAbs and Sm labeling reagent can be prolonged with a suitable excess of the Sm labeling reagents, which may help to get a higher Sm^{3+} label yield. Despite this, the detection sensitivity for PG Ⅰ with a limit of 0.2 μg/L is more than adequate for measuring the PG Ⅰ concentration in clinical samples.

Direct passive absorption of two or more binders is still the routine method for multi-analyte immunoassay (MAIA)[20]. When only the sandwich-type configuration is employed in MAIA, it is necessary to prepare an activated surface binder (e.g. antibody) in order to get a high sensitivity. In this study, the anti-PG Ⅰ and anti-PG Ⅱ antibodies were co-coated simultaneously in a strip well, which was found to be beneficial and economical for a suitable fluorescence by adjusting the concentration of coated antibodies. The total concentration of the co-coated antibodies which can achieve favorable results was no more than 5 μg/mL (1000 ng per well) in this study.

Samples with a relatively high PG Ⅰ or PG Ⅱ were analyzed at various dilutions. The diluting buffer was identical to the calibrator buffer. The percentage of expected value was 96.3%-101.7% for PG Ⅰ and 98.1%-109.6% for PG Ⅱ. No hook effect of dual-label assay was found at a relatively high PG Ⅰ or PG Ⅱ concentration. Recovery was identified by supplementing PG Ⅰ calibrators at 20 μg/L and 100 μg/L, and PG Ⅱ calibrators at 5 μg/L and 50 μg/L. The average recovery rates for PG Ⅰ and PG Ⅱ were 102.7% and 98.8%, respectively, showing that the analytical accuracy is satisfactory for clinical use.

Different analytes (i.e. t-PSA/f-PSA, AFP/CEA) can be detected at present by dual-label TRFIA[3,21] and the corresponding instruments are commercially available. In this study, the human PG detected by dual-label TRFIA was similar to that detected by ELISA and single-label TRFIA. The sensitivity, measurement range and stability of dual-label TRFIA were substantially better than those of ELISA. Unlike radioimmunoassay or ELISA[22,23], dual-label TRFIA can measure the concentration of PG Ⅰ and PG Ⅱ, as well as the ratio of PG Ⅰ/Ⅱ, thus reducing the random handling errors and increasing the clinical confidence level of PG Ⅰ/PG Ⅱ ratio. Direct labeling of immunoreagents with lanthanide chelates and lack of overlapping between Eu^{3+} and Sm^{3+} chelates allow a rapid assay. In addition, 25 μL of samples is enough for the simultaneously detection of PG Ⅰ and PG Ⅱ.

In summary, dual-label TRFIA can serve as a high-throughput tool for the detection of serum PG and has good prospects of clinical application.

COMMENTS

Background

Non-invasive serum pepsinogen (PG) test provides much information on intestinal metaplasia, atrophic gastritis, as well as *Helicobacter pylori* infection and peptide ulcer, which has a significant clinical value for the mass screening of patients at a high risk of gastric cancer. A reliable and effective detection method covering a wide concentration range with good sensitivity for PG is required. Furthermore, simultaneous determination of PG Ⅰ and PG Ⅱ can improve the confidence level of the PG Ⅰ/PG Ⅱ ratio.

Research frontiers

In this study, the authors found that multi-analyte immunoassay could increase the throughput and reduce the overall cost per test. A simple, rapid and sensitive dual-label time-resolved fluoroimmunoassay (TRFIA) for pepsinogens in human serum was developed.

Innovations and breakthroughs

TRFIA is a sensitive technique used in analysis of trace substances. Compared with traditional methods, such as radioimmunoassay or enzyme-linked immunosorbent assay (ELISA), dual-label assay can reduce random handling errors and increase the confidence level of PG Ⅰ and PG Ⅱ (especially for the PG Ⅰ/PG Ⅱ ratio). Only 25 μL of serum samples is enough for each test, which is useful for mass screening.

Applications

The PG detected by dual-label TRFIA was in good agreement with that detected by single-label assay or by ELISA. The analytical accuracy, precision and stability are satisfactory for its use in clinical practice. Dual-label TRFIA may serve as a high-throughput tool for the detection of serum PG and has good prospects of clinical application.

Peer review

The analysis of pepsinogens in serum/plasma of patients is a well established method to identify subjects at a higher risk of developing gastric cancer. The described method for analyzing Pep-Ⅰ and Pep-Ⅱ simultaneously seems to have similar parameters in relation to sensitivity and specificity as EIA or ELISA. The manuscript is well written.

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BRIEF ARTICLE

Construction and characterization of calreticulin-HBsAg fusion gene recombinant adenovirus expression vector

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Abstract

AIM: To generate recombinant adenoviral vector containing calreticulin (CRT)-hepatitis B surface antigen (HBsAg) fusion gene for developing a safe, effective and HBsAg-specific therapeutic vaccine.

METHODS: CRT and HBsAg gene were fused using polymerase chain reaction (PCR), endonuclease digestion and ligation methods. The fusion gene was cloned into pENTR/D-TOPO transfer vector after the base pairs of DNA (CACC) sequence was added to the 5′ end. Adenoviral expression vector containing CRT-HBsAg fusion gene was constructed by homologous recombinantion. The human embryo kidney (HEK) 293A cells were transfected with linearized DNA plasmid of the recombinant adenoviral vector to package and amplify recombinant adenovirus. The recombinant adenovirus titer was characterized using the end-dilution assay. The expression of the CRT/HBsAg fusion protein in Ad-CRT/HBsAg infected 293A cells was detected by Western blotting.

RESULTS: The CRT-HBsAg fusion gene was characterized by PCR and sequencing and its length and sequence were confirmed to be accurate. The CRT-HBsAg fusion gene recombinant pENTR/D-TOPO transfer vector was constructed. The recombinant adenoviral vector, Ad-CRT/HBsAg, was generated successfully. The titer of Ad-CRT/HBsAg was characterized as 3.9 × 10^{11} pfu/mL. The CRT-HBsAg fusion protein was expressed by HEK 293A cells correctly.

CONCLUSION: CRT/HBsAg fusion gene recombinant replication-defective adenovirus expression vector is constructed successfully and this study has provided an experimental basis for further studies of Hepatitis B virus gene therapy.

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Key words: Calreticulin; Hepatitis B virus; Hepatitis B surface antigen; Adenovirus expression vector; Fusion protein; Therapeutic vaccine

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INTRODUCTION

Development of a safe, effective and therapeutic vaccine represents the best hope for treatment of hepatitis B virus (HBV) infection. Recent data have indicated that immunotherapeutic strategies stimulating both cellular and humoral immune responses to HBV antigens are es-

sential to cure chronic HBV infection[1]. In this regard, DNA-based vaccination appears to be a particularly pertinent approach for chronic hepatitis B treatment, since it has been well documented to elicit durable humoral and cell mediated immunity including cytotoxic T lymphocytes and cytokines in normal mice[2,3], ducks[4] and chimpanzees[5]. It is a good candidate for immunization of non-responders to recombinant hepatitis B surface antigen (HBsAg) vaccines and for therapeutic vaccination[2]. However, even at higher doses of DNA, human clinical trials of HBV DNA vaccine have yielded a much lower level of immune responses than that observed in small animals[6,7]. Therefore, the potency of DNA vaccines must be enhanced for successful human application. The use of calreticulin (CRT) represents an innovative and feasible approach for enhancing immune responses and generating an antiangiogenic effect[8]. CRT is an abundant 46 kDa Ca^{2+}-binding protein located in the endoplasmic reticulum (ER)[9]. CRT is considered to be related to the family of heat shock proteins[10,11]. The protein has been shown to associate with peptides delivered into the ER by transporters associated with antigen processing (TAP-1 and TAP-2)[12] and with major histocompatibility complex (MHC) class I -β2 microglobulin molecules to aid in antigen presentation[13].

It is well known that available recombinant HBsAg protein vaccine is effective for preventing HBV infection for healthy persons, but for the infected patients there have been no effective and therapeutic vaccines until now. In this study, CRT-HBsAg fusion gene recombinant adenovirus expression vector was generated to establish the basis for curing chronic HBV infection and HBsAg-positive liver cancer.

MARTERIALS AND METHODS

Plasmids, cells and reagents

ViraPower Adenoviral Expression System including pENTR/D-TOPO Cloning Vector, Adenoviral Expression Vector, HEK293A cells, TOP10 competent cells of *Escherichia coli (E. coli)* were all purchased from Invitrogen Corporation (USA). The pJW4303, plasmid was provided by professor Lu Shan (Umass, USA).

Taq DNA polymerase, *Pfu* DNA polymerase, antibodies marked by HRP, *Pac*I, plasmid DNA purification kit and Gel purification kit were purchased from Tianwei Shidai Corporation (China), New England Corporation (UK), Takara Corporation (China), and Promega Corporation (USA), respectively. Human anti-HBsAg antibody positive serum was collected by our laboratory. Primers were synthesized in Shanghai Invitrogen Corporation (China).

Construction and preparation of CRT/HBsAg fusion gene recombinant plasmid

For the generation of pJW43033-CRT, CRT was amplified with polymerase chain reaction (PCR) using rabbit CRT cDNA as the template[14] and a set of primers, 5'-CCGGAGACTCATGCTGCTCCCTGTGCCGCT-3' and 5'-CCGGGAATTCCAGCTCGTCCTTGGCCTGGC-3'. There is more than 90% homology between rabbit, human, mouse, and rat CRT[14]. The amplified product was then cloned into the *Sac*I/*Eco*RI sites of pJW4303 vector. For the generation of pJW4303-CRT/HBsAg, HBsAg gene was amplified with a set of primers, 5'-GTGGGGAATTCATGGAGAACACAACATCAGG-3' and 5'-GGGGTGGATCCAATTTACATATGGGTTTCTGT-3', and then cloned into the *Eco*RI/*Bam*HI sites of pJW4303-CRT to generate pJW4303-CRT/HBsAg. The accuracy of these constructs was confirmed by endonuclease digestion and DNA sequencing. The sequencing was finished by Shanghai Invitrogen Biological Engineering Corp (China).

Construction of recombinant pENTR/D-TOPO transfer vector containing CRT/HBsAg fusion gene

CRT/HBsAg fusion gene was amplified using pJW4303-CRT/HBsAg plasmid as the template and a set of primers, 5'-CACCATGCTGCTCCCTGTGCCGCT-3' and 5'-AATTTACATATGGGTTTCTGT-3'. In order to construct directional TOPO cloning transfer vector in the desired direction, the CACC sequence was incorporated into the PCR upstream primer at its 5' end. The final optimized conditions of PCR consisted of 94℃ for 5 min, followed by 30 cycles of denaturation at 94℃ for 30 s, annealing at 55℃ for 30 s and extension at 72℃ for 1 min. After PCR, the PCR products of fusion gene were purified for generating the pENTR/D-TOPO transfer vector. The ligated products were transformed into TOP10 chemically competent *E. coli* and incubated overnight at 37℃. Subsequently, extracted and purified plasmids containing CRT/HBsAg fusion gene were characterized by PCR and sequencing, respectively. The sequencing was finished by Shanghai Invitrogen Biological Engineering Corp.

Construction and characterization of CRT/HBsAg fusion gene recombinant adenoviral expression vector, Ad-CRT/HBsAg

Using LR Clonase Mix™ (Invitrogen, US) as catalysis, the purified CRT/HBsAg fusion gene recombinant pENTR/D-TOPO transfer vector was mixed with adenoviral expression vector DNA plasmid to generate the recombinant adenoviral expression vector. The recombinant products were transformed into TOP10 chemically competent *E. coli* and incubated on Luria-Bertani (LB) plates containing 100 μg/mL ampicillin at 37℃ overnight. Subsequently, we selected eight putative positive clones which were ampicillin-resistant to amplify, extract and purify for PCR amplification and electrophoresis detection, respectively. The reason why using LB plates containing 30 μg/mL chloramphenicol to select positive clones is because true expression clones would be ampicillin-resistant and chloramphenicol-sensitive.

Transfecting the HEK 293A cells line with Ad-CRT/HBsAg plasmid

Purified Ad-CRT/HBsAg DNA plasmid was digested with *Pac*I. The day before transfection, the 293A cells were

trysinized, counted, and plated at 5×10^5 cells per well in a 6-well plate. Two milliliter of normally growing Dulbecco's modified Eagle's medium (D-MEM) containing 10% fetal bovine serum (FBS) was plated into each well. Those 293A cells were transfected using Lipofectamine 2000™ (Invitrogen, USA) and Pac I-digested expression plasmid DNA complexes. Culture medium was replaced with refresh complete culture medium every 2-3 d until visible regions of cytopathic effect (CPE) were observed. When approximately 80% of CPE was observed, adenovirus-containing cells and media were harvested.

LacZ gene recombinant adenoviral expression vector plasmid (Ad-LacZ) provided by the manufacturer (Invitrogen, USA) was used as control.

Characterization and titering of Ad-CRT/HBsAg

The titer of Ad-CRT/HBsAg was characterized using the End-Point Dilution Assay. When the 293A cells prepared 24 h before incubated at 37℃ in a CO_2 incubator were at more than 80% confluency in 96-well plates, the recombinant adenovirus stock solution harvested above was diluted to a concentration of 10^{-1}-10^{-12} and 100 μL was added to each well. After cultured for 10 d, the number of wells with CPE was calculated according to the formula of titre (pfu/mL) = $10^{(x + 0.8)}$.

Western blotting analysis

After being cultured for 72 h, the Ad-CRT/HBsAg infected and normal control 293A cells were harvested and frozen to -80℃. Some of them were prepared for Western blotting analysis. Protein samples were fractionated on 5%-12% SDS-PAGE and transferred to PVDF membrane using protein transfer apparatus (Bio-Rad, USA). The membrane was blocked for 1 h with 5% skimmed milk in TBS buffer (50 mmol/L Tris base, 150 mmol/L NaCl, pH 7.5) containing 0.5% Triton X-100. The membrane was probed with human anti-HBsAg antibody positive serum. After washing the membrane, goat anti-human HRP-conjugated antibody was added and incubated. The results were finally revealed using the sensitive substrate of DAB kit (Wuhan Boster, China) for Western blotting detection.

RESULTS

Characterization of CRT/HBsAg fusion gene recombinant plasmid

The CRT/HBsAg fusion gene recombinant plasmid pJW4303-CRT/HBsAg was digested by *Sac*I and *Bam*HI endonucleases. An approximately 2000 bp (1944 bp = 1263 bp + 681 bp) long DNA band was observed using 1.5% agarose gel electrophoresis (Figure 1A).

Characterization of CRT/HBsAg fusion gene recombinant pENTR/D-TOPO transfer vector

Kanamycin-resistant putative positive clones were prepared as template to amplify CRT/HBsAg fusion gene by specific PCR. Approximately 2000 bp long DNA bands were observed in those positive clones using 1.5% agarose gel electrophoresis (Figure 1B). The sequencing results of CRT and HBsAg gene were the same as CRT sequence of NM004343.3 and HBsAg sequence of AF013631 in GenBank.

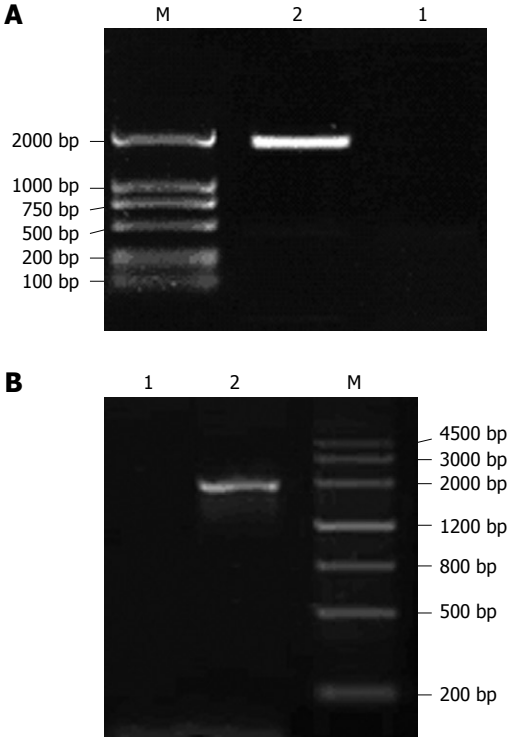


Figure 1 Characterization of polymerase chain reaction (PCR) amplification by electrophoresis. A: Amplification of calreticulin (CRT)/hepatitis B surface antigen (HBsAg) fusion gene by PCR. Lane 1: Negative control; Lane 2: CRT/HBsAg fusion gene recombinant pJW4303 plasmid; Lane M: DNA marker; B: Characterization of CRT/HBsAg fusion gene recombinant pENTR/D-TOPO transfer vector by PCR. Lane 1: Negative control; Lane 2: CRT/HBsAg fusion gene recombinant pENTR/D-TOPO transfer vector amplified by specific PCR; Lane M: DNA marker.

Characterization of CRT/HBsAg fusion gene recombinant adenoviral expression vector, Ad-CRT/HBsAg

All of the selected clones were sensitive for culturing in LB plates containing 30 μg/mL chloramphenicol because those true expression clones would be chloramphenicol-sensitive. After PCR amplification, a nearly 2000 bp long DNA band was observed using 1.5% agarose gel electrophoresis.

Harvest and detection of recombinant adenovirus, Ad-CRT/HBsAg

*Pac*I-digested Ad-CRT/HBsAg DNA plasmid was transfected into 293A cells by Lipofectamine 2000™. About 10 d after transfection, 80% of the cells rounded up and were floating or lightly attached to the tissue culture dish. This indicated that cells were loaded with adenovirus particles (Figure 2A). The shape of normal 293A cells are shown in Figure 2B. The adenovirus-containing cells were harvested and characterized by PCR amplification. A DNA band, which was approximately 2000 bp long, was found with 1.5% agarose gel electrophoresis.

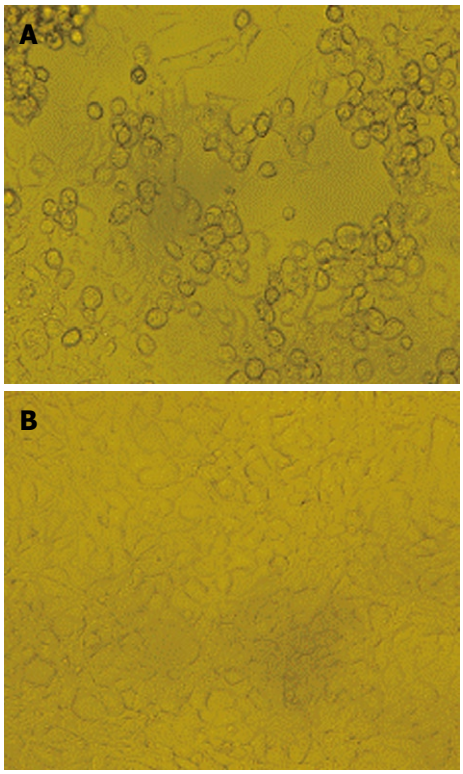


Figure 2 Culture of recombinant adenovirus in HEK293A cells. A: CPE was observed in recombinant adenovirus-containing HEK293A cells; B: Normal HEK293A cells cultured as control.

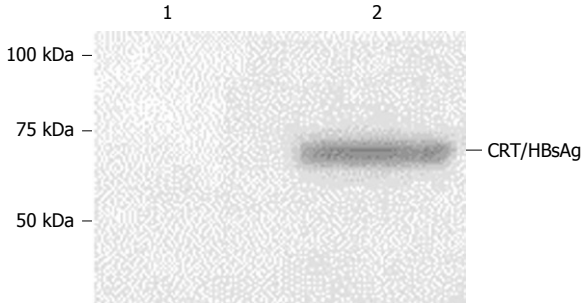


Figure 3 Characterization of CRT/HBsAg fusion protein expression by Western blotting. The expression of CRT/HBsAg fusion protein in 293A cells was determined in 293A cells transfected with Ad-CRT/HBsAg and Ad-LacZ by Western blotting analysis. Human anti-HBsAg positive serum was used at a 1:100 dilution for the detection of CRT/HBsAg fusion protein expression. Lane 1: Lysate from 293 cells transfected with Ad-LacZ; Lane 2: Lysate from 293 cells transfected with Ad-CRT/HBsAg.

Titering of the adenoviral stock, Ad-CRT/HBsAg

After having been amplified, the recombinant adenoviral stock was characterized by the End-Point Dilution Assay. Its titer was calculated as 3.9×10^{11} pfu/mL.

Characterization of CRT/HBsAg fusion protein expression

The expression of CRT/HBsAg fusion protein in Ad-CRT/HBsAg infected 293A cells was detected by Western blotting. Figure 3 shows the 71 kDa CRT/HBsAg fusion protein expression.

DISCUSSION

There are approximately four hundred million chronic hepatitis B patients in the world, and effective therapeutic vaccines are needed urgently. The purpose of this study is to facilitate the development of a new type of therapeutic HBV vaccine. The research hotspot in this field is how to develop an effective therapeutic vaccine by modifying the HBV antigens such as HBsAg, pres1Ag, pres2Ag and HBcAg to induce a high level of humoral and cellular immune responses for clearing the HBV infection. The HBV therapeutic vaccines include protein vaccine, DNA vaccine and virus vector vaccine, but none of them has been developed successfully.

Recently, investigators have performed a head-to-head comparison of linkage of antigen to CRT in a DNA vaccine, and achieved the greatest enhancement of the humoral and T-cell-mediated immune responses in vaccinated mice[15]. Previous studies have shown that adenoviral vectors expressing the fusion of CRT and E7 can effectively be used as a prophylactic and therapeutic vaccine against E7-expressing tumors[16]. In this study, the HBV surface gene was fused with CRT, which can improve immunogenicity by enhancing the MHC class I presentation of linked peptide/protein. CRT-HBsAg fusion gene recombinant adenovirus vector vaccine will be an ideal candidate for HBV therapeutic vaccine, because it can induce HBsAg specific high-level immune responses.

Members of adenovirus family infect a great variety of post-mitotic cells, even those associated with highly differentiated tissues such as skeletal muscle, lung, brain and heart. This characteristic, together with being relatively easy for preparation and purification, has led to their extensive use as gene vectors. Since they deliver their genome to the nucleus and can replicate with high efficiency, they are prime candidates for the expression and delivery of therapeutic genes[17]. The adenovirus vectors used in this study are to be utilized for delivering genes of CRT fused HBsAg to liver tissues to suppress and eliminate chronic inflammation and tumors caused by HBV infection. This is for the first time to construct the CRT-HBsAg gene recombinant adenovirus as a therapeutic vaccine. This study suggested that CRT-HBsAg fusion gene recombinant adenovirus vector may be a potent tool for treating chronic HBV carriers and liver cancer patients.

COMMENTS

Background

The hepatitis B virus (HBV) infection is one of the most widespread viral infections of human and causes acute and chronic hepatitis and hepatocellular carcinoma. Problem of HBV infection has necessitated the development of an effective therapeutic vaccine. Calreticulin (CRT) is a ubiquitously expressed Ca^{2+} binding protein in endoplasmic reticulum of eukaryotic cells. Previous studies have shown that CRT enhances the major histocompatibility complex (MHC) class I presentation of linked peptide/protein and may serve as an effective vaccination strategy for antigen-specific cancer treatment. In this study, CRT-hepatitis B surface antigen (HBsAg) fusion gene recombinant adenovirus

expression vector was generated to establish the basis for curing acute and chronic HBV infection and HBsAg-positive hepatocellular carcinoma.

Research frontiers

There are approximately four hundred million chronic hepatitis B patients in the world, and effective therapeutic vaccines are needed urgently. The research hotspot is how to develop an effective therapeutic vaccine by modifying the HBV antigens such as HBsAg, pres1Ag, pres2Ag and HBcAg to induce a high level of humoral and cellular immune responses for clearing the HBV infection. HBV therapeutic vaccines include protein vaccine, DNA vaccine and virus vector vaccine, but none of them has been developed successfully.

Innovations and breakthroughs

Most of the previous researches about HBV therapeutic vaccines focus on modifying HBsAg to enhance its immunogenicity. In this study, the HBV surface gene was fused with CRT, which can improve immunogenicity by enhancing the MHC class I presentation of linked peptide/protein. CRT-HBsAg fusion gene recombinant adenovirus vector vaccine will be an ideal candidate for HBV therapeutic vaccine, because it can induce HBsAg specific high-level immune responses. Adenovirus can infect a great variety of cell types and tissues in both dividing and non-dividing cells. This characteristic, together with being relatively easy for preparation and purification, has led to their extensive use as gene vectors. The adenovirus vectors used in this study are to be utilized for delivering genes of CRT fused HBsAg to liver tissues to suppress and eliminate tumors and inflammation caused by HBV infection. This is for the first time to construct the CRT-HBsAg gene recombinant adenovirus as a therapeutic vaccine.

Applications

CRT linked HBsAg gene recombinant adenovirus vectors were constructed in this study to develop a new sort of HBV therapeutic vaccine for the purpose of curing chronic hepatitis and hepatocellular carcinoma.

Peer review

It is a nice work to construct CRT/HBsAg fusion gene recombinant replication-defective adenovirus expression vector and it has provided a therapeutic vaccine and an experimental basis for further research of HBV gene therapy.

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CASE REPORT

Obstructing fungal cholangitis complicating metal biliary stent placement in pancreatic cancer

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Abstract

Biliary obstructions can lead to infections of the biliary system, particularly in patients with occluded biliary stents. Fungal organisms are frequently found in biliary aspirates of patients who have been on antibiotics and have stents; however, fungal masses, or "balls", that fully obstruct the biliary system are uncommon and exceedingly difficult to eradicate. We present 4 cases of obstructing fungal cholangitis in patients who had metal biliary stents placed for pancreatic malignancies, and subsequently required aggressive antifungal administration along with endoscopic and radiologic interventions. This report also reviews approaches previously undertaken to manage severe obstructing fungal cholangitis.

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Key words: Obstructing fungal cholangitis; Biliary stents; Fungal balls; Pancreatic cancer; Biliary obstruction

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Story B, Gluck M. Obstructing fungal cholangitis complicating metal biliary stent placement in pancreatic cancer. *World J Gastroenterol* 2010; 16(24): 3083-3086 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i24/3083.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i24.3083

INTRODUCTION

Obstructing lesions of the biliary tree include benign and malignant strictures, bile duct stones, stenotic sphincters, and obstructed endoprostheses. Obstructions can lead to infections of the biliary system, particularly in patients with occluded biliary stents. Fungal organisms are frequently found in biliary aspirates of patients who have been on antibiotics and have stents; however, fungal masses, or "balls", that fully obstruct the biliary system are uncommon and exceedingly difficult to eradicate[1-3]. We present 4 cases of obstructing fungal cholangitis in patients who had metal biliary stents placed for pancreatic malignancies and subsequently required aggressive endoscopic and radiologic interventions.

CASE REPORT

Case 1

A 48-year-old male with locally advanced unresectable pancreatic cancer on gemcitabine and docetaxel chemotherapy, presented to our institution with fevers, rigors, jaundice, and epigastric pain 7 d after placement of a metal biliary stent at another institution. Antibiotics were started and endoscopic retrograde cholangiopancreatography (ERCP) showed multiple large filling defects resembling stones (Figure 1). The bile duct was cleared of all defects and the biliary stent was exchanged for a new longer metal biliary stent. Biliary aspirates grew *Candida albicans*. Despite starting 200 mg daily intravenous (iv) fluconazole, the biliary tree rapidly reoccluded within 3 d with large fungal balls. A repeat ERCP in which a plastic

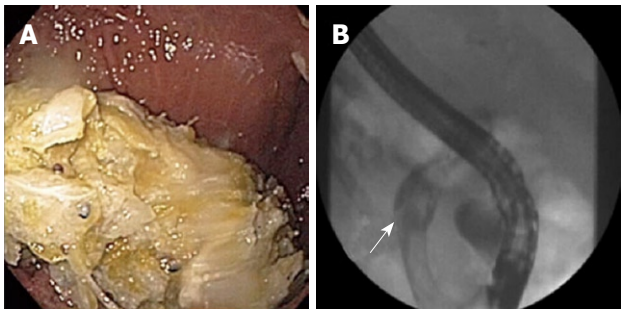


Figure 1 Endoscopic and cholangiographic views of biliary obstruction in patient 1. A: Biliary/fungal debris occluding metal biliary stent; B: "Fungal balls" (arrow) obstructing the bile ducts by cholangiography.

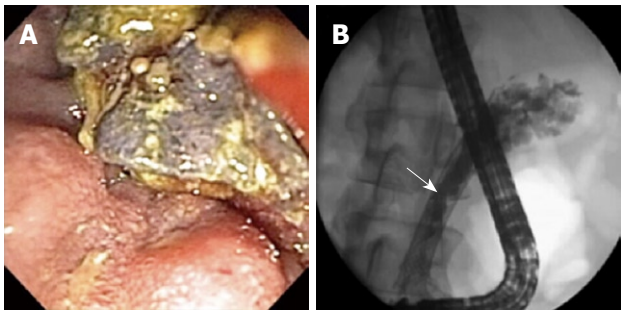


Figure 2 Endoscopic and cholangiographic views of biliary obstruction in patient 2. A: "Fungal ball" visible in the common bile duct; B: Cholangiogram showing fungal debris (arrow) around the metal biliary stent.

endoprosthesis was placed also occluded within 5 d with large fungal balls. A percutaneous transhepatic biliary drain (PTBD) was placed and twice daily 100 mg fluconazole flushes were initiated through the drain. The IV fluconazole was switched to 50 mg daily iv caspofungin, and this antifungal combination resulted in no further obstructions. The patient was discharged home on 200 mg/d oral fluconazole and twice daily 100 mg fluconazole flushes to be continued indefinitely. At 2 mo, repeat biliary cultures through the PTBD identified fluconazole-resistant *Candida*, prompting the patient's oral regimen to be switched to 200 mg twice daily oral voriconazole. The remainder of the patient's clinical course required occasional PTBD catheter exchanges, daily oral voriconazole, and daily intrabiliary flushing of fluconazole until he died 5 mo later secondary to his advanced pancreatic cancer.

Case 2

A 66-year-old female with metastatic pancreatic cancer on gemcitabine chemotherapy underwent metal biliary stent placement and percutaneous cholecystostomy tube placement for obstructive jaundice and acute cholecystitis. Her symptoms improved and 3 mo later, the gallbladder was removed. Shortly after surgery, she was readmitted for fevers, chills, and recurrent jaundice. Antibiotics for presumptive bacterial cholangitis were started and an ERCP was performed revealing caked debris on the proximal edge of her metal biliary stent and multiple soft filling defects within the common hepatic duct (Figure 2). Biliary aspirates grew *Candida glabrata* and *Candida albicans*. Despite clearing the duct of all debris and starting 200 mg twice daily oral voriconazole and 100 mg twice daily fluconazole flushes through the cholecystostomy tube, her biliary tree rapidly reoccluded in 10 d with large fungal balls. A nasobiliary drain was then placed to further facilitate fluconazole flushing into the biliary system. Eradication was finally achieved with daily oral voriconazole and daily fluconazole flushing through both the cholecystostomy tube and the nasobiliary tube. On this regimen indefinitely, the patient remained clear of further obstructions and died from her advanced pancreatic cancer 8 mo later.

Case 3

A 56-year-old male with a Merkel cell lymphoma of his leg and a large metastasis to the pancreatic head, presented to our institution with increasing fevers, abdominal pain, nausea, vomiting, and jaundice. He had been undergoing chemotherapy with irinotecan and carboplatin, and had required duodenal stent placement for tumor overgrowth as well as PTBD and percutaneous metal biliary stent placement 7 mo previously for biliary obstruction. At the time of admission, empiric antibiotic therapy was initiated for presumptive bacterial cholangitis, and an upper endoscopy was performed revealing gastric outlet obstruction with tumor overgrowth of the duodenal stent. A new duodenal stent was placed but the biliary stent could not be visualized, requiring repeat PTBD placement. A PTBD tube check 5 d later revealed multiple soft filling defects within the PTBD catheter consistent with fungal balls (Figure 3). The PTBD catheter was exchanged and after biliary aspirates grew *Candida* spp, 400 mg/d oral fluconazole and 100 mg fluconazole flushes twice daily were initiated through the new PTBD catheter. However, after fluconazole-resistant *Candida glabrata* and *Candida albicans* were isolated from culture, antifungals were switched to 50 mg iv caspofungin once daily and 100 mg amphotericin B flushes once daily through the PTBD catheter. The patient was subsequently discharged from the hospital on a 3-wk course of this antifungal regimen. One month after completion of antifungal therapy, there was no further recurrence of biliary obstruction.

Case 4

A 64-year-old male with metastatic pancreatic cancer on gemcitabine and docetaxel chemotherapy, with an indwelling metal biliary stent in place, presented to an outside facility with biliary obstruction and bacterial cholangitis. Symptoms resolved after a PTBD was placed and antibiotics were administered. However, 2 wk later, the patient re-presented to our institution with recurrent fevers and jaundice. Empiric antibiotic therapy was again initiated for presumptive bacterial cholangitis. Cholangiography performed during ERCP demonstrated obstruction due to migration of the metal biliary stent, tumor overgrowth and multiple soft filling defects consistent with fungal balls. Exchange of the PTBD catheter allowed for external drainage of biliary debris, and the aspirate grew *Candida albicans* in culture. Intravenous fluconazole

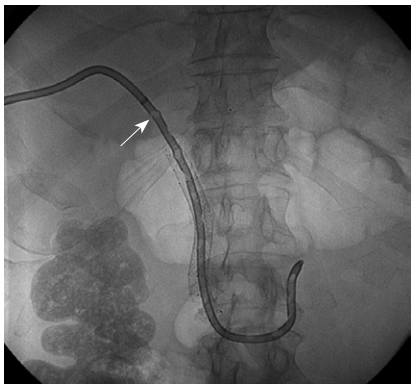


Figure 3 Cholangiographic view of fungal balls (ex. arrow) within the percutaneous transhepatic biliary drainage catheter in case 3.

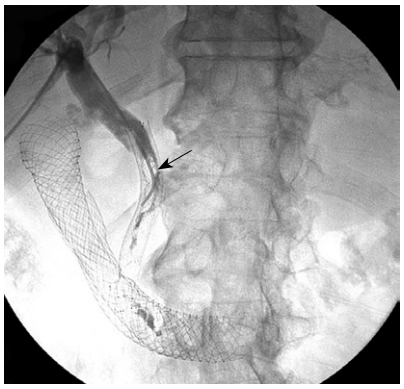


Figure 4 Cholangiographic view of fungal debris (arrow) within a metal biliary stent in case 4.

Table 1 Obstructing fungal cholangitis, summary of the literature

Author	Presentation	Treatment	Fungal eradication
Marcucci *et al*[1], 1978	Abdominal (Abd) pain, jaundice	Surgical removal of fungal balls/drainage. No chemotherapy	Yes
Magnussen *et al*[11], 1979	Acute myelogenous leukemia, elevated liver function tests (LFTs)	Surgical removal of fungal balls, amphotericin B	Yes
Carstensen *et al*[2], 1986	Abd pain, jaundice	Surgical removal, T-tube drainage, antifungals	Yes
Irani *et al*[7], 1986	Jaundice, s/p thoracic abdominal aortic aneurysm repair	Amphotericin B	No, died of cerebral infarct
Ho *et al*[3], 1988	Sickle cell disease, abd pain, jaundice, s/p cholecystecomy	Surgical removal, intrabiliary amphotericin B	Yes
Wig *et al*[4], 1998	Abd pain, jaundice	Nasobiliary drainage, ex-lap with T-tube placement; no chemotherapy	Yes
Reeves *et al*[8], 2000	Abd pain, jaundice	Endoscopic debridement, intrabiliary amphotericin B, oral fluconazole	Yes
Domagk *et al*[5], 2001 and	Elevated LFTs, dilated bile ducts	Sphincterotomy, debridement, systemic/intrabiliary antifungals	Yes
Domagk *et al*[6], 2006	Dilated bile ducts	Sphincterotomy, debridement, systemic/intrabiliary antifungals	Yes
	Abd pain, jaundice	Sphincterotomy, nasobiliary drainage, systemic/intrabiliary antifungals	Yes
	Abd pain, jaundice	Sphincterotomy, debridement, nasobiliary drainage, systemic/intrabiliary antifungals	Yes
	Elevated LFTs	Sphincterotomy, debridement, nasobiliary drainage, systemic/intrabiliary antifungals	Yes
	Elevated LFTs	Sphincterotomy, debridement, nasobiliary drainage, systemic/intrabiliary antifungals	No
	Abd pain, jaundice	Sphincterotomy, debridement, nasobiliary drainage, systemic/intrabiliary antifungals	No

200 mg/d was initiated for antifungal coverage. Over the next several days, biliary obstruction persisted due to stent malpositioning, which ultimately was relieved by severing the patient's migrated metal biliary stent. Despite these measures, within 5 d of PTBD catheter exchange and trimming of the metal biliary stent, the patient's bile duct reobstructed with fungal balls visualized on repeat tube check (Figure 4). The PTBD catheter was exchanged and the patient was initiated on an indefinite regimen of 200 mg oral voriconazole daily and 100 mg fluconazole flushes through the PTBD catheter twice daily. Two weeks later, the patient had no further evidence of obstruction.

DISCUSSION

The patients described in this case report highlight the difficulties in treatment of obstructing fungal cholangitis following the placement of metal biliary stents. Clinical improvement was not seen with endoscopic maneuvers alone or in combination with initial systemic antifungals. Ultimately, infection control was only achieved after flushing antifungals through percutaneous intrabiliary or nasobiliary drains and escalating systemic antifungal therapy from fluconazole to voriconazole or caspofungin. All patients remained free of *Candida* infection following optimization of their antifungal regimens.

While the presence of biliary stents likely played a role in the development of fungal cholangitis in our case report, there are several other factors that can contribute to the development of fungal infections. These include immunosuppression secondary to chemotherapy agents, malignant hematologic disease, broad-spectrum antibiotic administration, corticosteroid therapy, diabetes mellitus, improper sterilization of endoscopes or prostheses, previous surgery, or trauma[1-6]. The patients in our case study demonstrated a number of these risk factors; all were

treated with broad-spectrum antibiotics, all were immunocompromised secondary to systemic chemotherapy for their pancreatic malignancies, and all had been given intermittent corticosteroids for chemotherapy administration.

The data regarding optimal treatment strategies for management of obstructing fungal masses is limited. To our knowledge, only 13 cases of obstructing fungal cholangitis have been described in the literature, the majority of which underwent systemic and intrabiliary infusion of antifungals. Several studies support our treatment approach of initiating therapy with fluconazole, given its favorable efficacy toward *Candida albicans* and relatively few side effects[4,6]. The literature further supports the use of escalation of therapy to voriconazole or caspofungin[6] for infection control. Other case studies, prior to the availability of voriconazole, utilized amphotericin B or liposomal amphotericin[5-8]. While the use of systemic amphotericin has been shown to have numerous complications, several case reports demonstrated that local intrabiliary infusion could be effective[5,6,8]. Interestingly, 2 case studies[1,4] reported no further antifungal administration was necessary following bile duct drainage of fungal balls for complete fungal eradication. Further details are shown in Table 1.

Fungal biliary aspirates are frequently positive in patients who have stents and have been on antibiotics[9,10]. However, it is uncommon to find completely obstructed bile ducts caused by large fungal "balls," implying that growth of the organism was rapid in a previously contaminated system due to foreign bodies and antibiotics. The pathogenesis of common bile duct fungal balls is largely unknown; however, 2 possible explanations exist. Firstly, formation of microabscesses in the liver parenchyma from fungal sepsis may allow movement of fungal organisms into the extra-hepatic biliary system[11]. A second hypothesis involves direct invasion of *Candida* into the common bile duct from the duodenum, with "fungal ball" formation resulting from rapid growth of a superficial mat of fungal mycelia on the bile duct surface[7,11]. Fragmented pieces of fungi and mucoid debris become molded to bile duct walls. Subsequent invasion and proliferation of fungal hyphae may be further aided by the presence of metal biliary stents, acting as a scaffold for infection[7,11].

The treatment strategies described herein address both of the above hypotheses. Systemic antifungal administration targets fungal sepsis, preventing further aggregation of fungal organisms. The direct invasion hypothesis is supported by the fact that fungal balls are easily swept out of the biliary system by endoscopic maneuvers. As such, treating the fungal conglomerates directly by flushing antifungal medications through percutaneous or nasobiliary drains may be a more effective treatment strategy than systemic hematologic dissemination of antifungal therapies[8]. The results seen in the 4 cases described in this report further support this idea. While it seems intuitive that intrabiliary antifungal concentrations would be higher with direct flushing than with systemic antifungal administration, both approaches seem to be required to prevent reocclusion of the biliary system. Further studies are needed to expand on these findings.

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CASE REPORT

Stone extraction balloon-guided repeat self-expanding metal stent placement

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Abstract

Self-expanding metal stent (SEMS) placement offers safe and effective palliation in patients with upper gastrointestinal obstruction due to a malignancy. Well described complications of SEMS placement include tumor growth, obstruction, and stent migration. SEMS occlusions are treated by SEMS redeployment, argon plasma coagulation application, balloon dilation, and surgical bypass. At our center, we usually place the second SEMS into the first SEMS if there is complete occlusion by the tumor. We discovered an unusual complication during SEMS redeployment. The guidewire passed through the mesh of the first SEMS and caused the second SEMS to become entangled with the first SEMS. This led to the distortion and malfunction of the second SEMS, which worsened the gastric outlet obstruction. For lowering the risk of entanglement, we studied stone extraction balloon-guided repeat SEMS placement. This is the first report of a SEMS entangled by the mesh of the first SEMS and stone extraction balloon-guided repeat SEMS placement for lowering the risk of this complication.

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Key words: Gastric outlet obstruction; Self-expanding metal stent

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INTRODUCTION

In patients presenting with gastric outlet obstruction, upper gastrointestinal malignancies are the source in up to 39% of cases[1]. Surgical resection is the standard of care in patients who lack significant comorbidities[2]. In unresectable patients, gastrojejunostomy remains the standard of care if a surgical intervention is to be undertaken. In those patients with significant comorbidities, the morbidity rate approaches 40%[3], encouraging alternatives to surgery. In patients who are not surgical candidates, enteral stenting offers an attractive option[4]. Self-expanding metal stent (SEMS) has proven itself to be a safe and relatively cost-effective alternative to surgical palliation allowing the patient to be discharged and start postoperative (PO) intake earlier[5]. Complications related to SEMS placement include stent migration, managed by removal of the original SEMS and replacement with a new SEMS, and obstructions due to local disease progression, treated respectively with repeat SEMS placement, argon plasma coagulation application, balloon dilation, and surgical bypass[2]. We usually perform repeat SEMS placement when there is an obstruction after first stenting. Overlapping the second SEMS into the first SEMS is a safe and easy way for resolving stent obstruction. However, repeat SEMS placement can cause a serious problem if a guidewire transverses the mesh of

the first SEMS and the second SMES is deployed in the mesh of the first SEMS. It must be extremely rare and happens in specific conditions; we experienced just one case of this serious complication. However, once it has happened, this causes irreversible SEMS entanglement leading to further obstruction. Therefore, it is necessary to lower the risk of SEMS entanglement during repeat SEMS placement. To prevent a guidewire from passing through the mesh of the first SEMS, we devised our stone extraction balloon-guided repeat SEMS placement and applied it to one case successfully.

CASE REPORT

A seventy-year-old woman received an 80 mm long SEMS (Niti-S™ Pyloric uncovered stent, Taewoong Medical, Ilsan, Korea) for relieving gastric outlet obstruction due to advanced gastric cancer. Six months later, she presented with vomiting and abdominal distention. Investigation revealed tumor overgrowth distal to the placed SEMS. We decided to deploy another 120 mm long uncovered SEMS (Niti-S™ Pyloric covered stent, Taewoong Medical, Ilsan, Korea), overlapping the previous one. An endoscopy did not pass the existing stent because the proximal part of the existing SEMS was narrow. Thus, we advanced a guidewire from the proximal part of the first SEMS. After performing repeat SEMS placement, we encountered an unusual complication. The newly deployed SEMS was trapped and unexpanded at the entangled site (Figure 1). The guidewire passed through the mesh of the existing stent and caused the second SEMS to be trapped by the mesh of the first one. Because of this complication the gastric outlet obstruction was not improved. This painful experience made us think of stone extraction balloon-guided repeat SEMS placement to avoid SEMS entanglement in specific conditions; the insertion of a guidewire from the proximal or middle part of the first SEMS guided only by fluoroscopy and the acute angulation of duodenum. Several months later, we faced a similar situation. An eighty-four-year-old woman with an 80 mm long SEMS (Niti-S™ Pyloric uncovered stent, Taewoong Medical, Ilsan, Korea) presented with gastric outlet obstruction symptoms. Investigation found that tumor ingrowth had caused SEMS occlusion. We decided to deploy a 100 mm long covered SEMS (Niti-S™ Pyloric covered stent, Taewoong Medical, Ilsan, Korea) into the existing one. Endoscopy was advanced to the middle part of the first SEMS where tumor ingrowth caused near total occlusion. We used a 15 mm stone extraction balloon (ESCORT II™, Willson Cook, Winston-Salem, USA) before advancing a guidewire. An inflated 15 mm stone extraction balloon was passed through from the middle to the distal part of the existing stent without falling into the mesh and led a guidewire to reach the target area safely (Figures 2 and 3). Repeat SEMS placement was successfully performed without the misguidance of a guidewire (Figure 4).

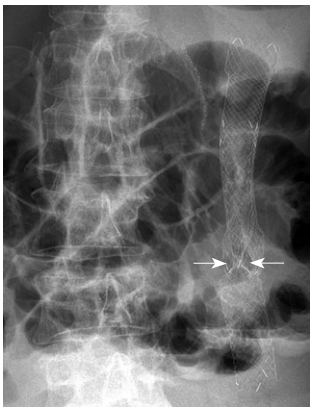


Figure 1 Plain film of the abdomen seven days after deploying the second self-expanding metal stent (SEMS). The unexpanded part of the second SEMS due to entanglement (arrows).

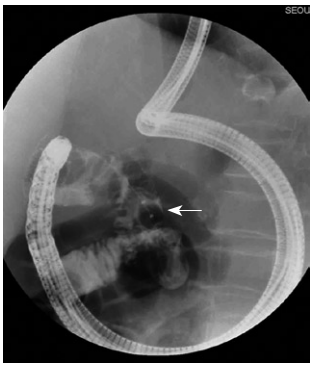


Figure 2 Fluoroscopy. The stone extraction balloon (arrow) was advanced distal to the existing SEMS.

DISCUSSION

The endoscopic placement of enteral SEMS relieves malignant gastroduodenal obstruction in the majority of patients, allowing discharge from hospital and the resumption of enteral nutrition[6]. A multi-center trial of patients with gastric outlet obstruction due to malignancy has concluded that enteral stent insertion allows a purely enteral diet to be maintained in up to 92% of patients, with 73% tolerating solid or semi-solid food[7]. Overall, if migration, fracture, tumor ingrowth, erosion, and perforation are taken into account, the global long-term patency rate obtained is 76%[2]. Stent migration occurs more often with covered stents since tumor ingrowth and subsequent incorporation of the stent into the surrounding tissue is less likely. In contrast, recurrent obstruction is more problematic for uncovered stents[8]. About 9% of total patients who received SEMS developed stent occlusion by local tumor growth with progression of the primary disease process[2]. These were treated, respectively, with repeat SEMS placement, argon plasma coagulation application, balloon dilation, and surgical bypass[2]. At our center, we usually perform repeat SEMS placement when there is a stent occlusion by tumor growth. We have performed repeat SEMS placement through endoscopic-fluoroscopic positioning. An endoscope with a working channel is placed at the distal portion of the first SEMS. Under fluoroscopic guidance, a guidewire pre-loaded through a catheter is used to gently cannulate the stenosis. Once the guidewire has passed through the stenosis, the catheter is advanced over the guidewire through the lesion. As soon as the catheter is passed through the stenosis, the guidewire is

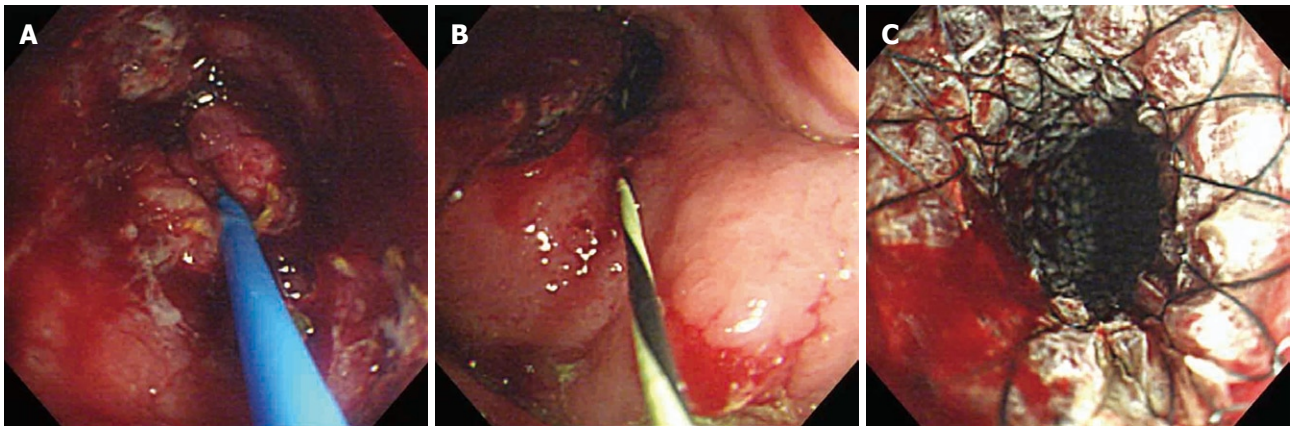


Figure 3 Duodenoscopy. A: The stone extraction balloon advancing over the region of tumor ingrowth; B: The guidewire advancing through the stone extraction balloon; C: The expanded covered SEMS.

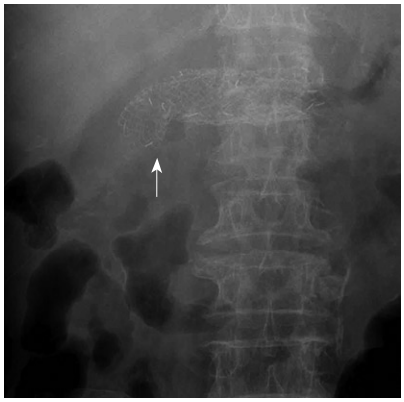


Figure 4 Plain film of the abdomen seven days after replacement of the SEMS. The fully expanded second SEMS overlapping the first SEMS without entanglement (arrow).

removed and water soluble contrast medium is injected to document the characteristics and length of the obstruction and to rule out the occurrence of perforation. At this point, it is very important to push and pull a guidewire through the catheter to find the right position. The stent apparatus is passed through the scope, advanced over the first SEMS under endoscopic and fluoroscopic guidance and deployed so that the middle of the stent covers the first SEMS with 2-2.5 cm extending beyond the proximal and distal margins of the obstructing neoplasm.

Repeat SEMS placement is generally safe and does not cause complications. However, in a specific condition, this procedure can result in irreversible SEMS entanglement. SEMS entanglement can happen by the combination of two conditions: acute angulation of the duodenum and stenosis of the first SEMS that prohibits an endoscope from passing through it. Stenosis of the first SEMS makes practice totally dependent on fluoroscopy, and acute angulation causes a guidewire to touch the duodenal wall. These two conditions make it possible for a guidewire to pass through the mesh of the first SEMS. It is definite that the possibility for a guidewire to transverse the mesh depends more on the angle of a guidewire direction to the mesh wall than on the catheter diameter. Because of the acute angulation of the SEMS in the duodenum, this situation can happen in duodenal obstruction treated by repeat SEMS placement, with frequent misinsertion of the guidewire. Although the acute angulation of an SEMS and the duodenum is the major factor responsible for SEMS entanglement, we thought that a thin guidewire was also responsible for this complication. Because the tip of the guidewire is smaller than the pores of the mesh, it can transverse the mesh and misguide the second SEMS under these specific conditions. When it does occur, the entanglement leads the second SEMS remaining unexpanded and causes further occlusion. For avoiding entanglement of two SEMSs, it is important to push forward and pull back a guidewire repeatedly to ensure the correct position of the guidewire, before starting the insertion of a second SEMS. It is also necessary for the deployment of the new stent to be slow and under careful radiologic control, because most stents can be recovered in their own sheath during the deployment maneuvers, when difficulties are encountered. Adding to these important skills, using a stone extraction balloon can be considered instead of using an ordinary guidewire. When inflated, this system is larger than the pores of the mesh, and therefore, the stone extraction balloon is less likely to pass through the mesh. We used three steps to perform stone extraction balloon-guided repeat SEMS placement in the specific conditions mentioned before. First, we advanced the stone extraction balloon distal to the first SEMS. Second, we inserted the guidewire through the stone extraction balloon so that it was placed at the target area. Finally, we placed the second SEMS within the first one following the guidewire. When there was an acute angulation, and an endoscope was not able to pass through the first SEMS, it seemed easier and safer to perform repeat SEMS placement by the guidance of a stone extraction balloon under careful radiologic control with repeat push and pull of a guidewire.

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Meetings

Events Calendar 2010

January 25-26
Tamilnadu, India
International Conference on Medical Negligence and Litigation in Medical Practice

January 25-29
Waikoloa, HI, United States
Selected Topics in Internal Medicine

January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology Conference

January 28-30
Hong Kong, China
The 1st International Congress on Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of Gastroenterology & Endoscopy Meeting

March 09-12
Brussels, Belgium
30th International Symposium on Intensive Care and Emergency Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian National Association for Study of the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on Diabetes PACD14

March 25-28
Beijing, China
The 20th Conference of the Asian Pacific Association for the Study of the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology and Hepatology Conference, EGHC 2010

April 14-17
Landover, Maryland, United States
12th World Congress of Endoscopic Surgery

April 14-18
Vienna, Austria
The International Liver Congress™ 2010

April 28-May 01
Dubrovnik, Croatia
3rd Central European Congress of surgery and the 5th Croatian Congress of Surgery

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual Meeting

May 06-08
Munich, Germany
The Power of Programming: International Conference on Developmental Origins of Health and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and Rectal Surgeons Annual Meeting

June 04-06
Chicago, IL, United States
American Society of Clinical Oncologists Annual Meeting

June 09-12
Singapore, Singapore
13th International Conference on Emergency Medicine

June 14
Kosice, Slovakia
Gastro-intestinal Models in the Research of Probiotics and Prebiotics-Scientific Symposium

June 16-19
Hong Kong, China
ILTS: International Liver Transplantation Society ILTS Annual International Congress

June 20-23
Mannheim, Germany
16th World Congress for Bronchoesophagology-WCBE

June 25-29
Orlando, FL, United States
70th ADA Diabetes Scientific Sessions

August 28-31
Boston, Massachusetts, United States
10th OESO World Congress on Diseases of the Oesophagus 2010

September 10-12
Montreal, Canada
International Liver Association's Fourth Annual Conference

September 11-12
La Jolla, CA, United States
New Advances in Inflammatory Bowel Disease

September 12-15
Boston, MA, United States
ICAAC: Interscience Conference on Antimicrobial Agents and Chemotherapy Annual Meeting

September 16-18
Prague, Czech Republic
Prague Hepatology Meeting 2010

September 23-26
Prague, Czech Republic
The 1st World Congress on Controversies in Gastroenterology & Liver Diseases

October 07-09
Belgrade, Serbia
The 7th Biannual International Symposium of Society of Coloproctology

October 15-20
San Antonio, TX, United States
ACG 2010: American College of Gastroenterology Annual Scienitfic Meeting

October 23-27
Barcelona, Spain
18th United European Gastroenterology Week

October 29-November 02
Boston, Massachusetts, United States
The Liver Meeting® 2010--AASLD's 61st Annual Meeting

November 13-14
San Francisco, CA, United States
Case-Based Approach to the Management of Inflammatory Bowel Disease

December 02-04
San Francisco, CA, United States
The Medical Management of HIV/AIDS

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Acknowledgments

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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

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/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

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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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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