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EDITORIAL

Minimally invasive surgery for esophageal achalasia

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Abstract

Esophageal achalasia is the most commonly diagnosed primary esophageal motor disorder and the second most common functional esophageal disorder. Current therapy of achalasia is directed toward elimination of the outflow resistance caused by failure of the lower esophageal sphincter to relax completely upon swallowing. The advent of minimally invasive surgery has nearly replaced endoscopic pneumatic dilation as the first-line therapeutic approach. In this editorial, the rationale and the evidence supporting the use of laparoscopic Heller myotomy combined with fundoplication as a primary treatment of achalasia are reviewed.

Key words: Esophagus; Achalasia; Laparoscopy; Heller myotomy; Gastroesophageal reflux

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INTRODUCTION

Idiopathic achalasia is a primary motor disorder characterized by incomplete relaxation of the lower esophageal sphincter and aperistalsis of the esophageal body secondary to loss of inhibitory ganglion cells in the myenteric plexus. It is unclear whether the primary event occurs in the brain or whether the neurologic changes are the result of a direct injury of the myenteric plexus. The etiology of the disease is unknown, with genetic, autoimmune, infectious, and environmental factors being implicated. Achalasia, usually diagnosed between 20 and 40 years or after 60 years of age, is the most common primary esophageal motor disorder; it is second only to gastroesophageal reflux disease as the most common functional esophageal disorder to require surgical intervention. The first account of a successfully treated case of achalasia was described by Thomas Willis in 1674, in which a whale bone was used to forcibly dilate the cardia. The first surgical myotomy was performed by Ernst Heller in 1913.

PATHOPHYSIOLOGY AND ASSESSMENT OF ACHALASIA

Failure of the lower esophageal sphincter to completely relax upon swallowing results in a functional obstruction and pressurization of the esophageal body. Defective esophageal emptying progressively leads to dilatation and tortuosity of the esophagus with loss of the peristaltic waveform. Overall deterioration of esophageal function and structure with time, and the fact that peristalsis can return after surgical myotomy, suggest that the motor abnormalities secondary to esophageal outflow obstruction may be reversible and that early definitive treatment of achalasia is essential to preserve esophageal function[1].

Achalasia has an insidious onset. Dysphagia and food regurgitation are the two major presenting symptoms of the disease. Nocturnal regurgitation often leads to recurrent episodes of aspiration pneumonia. In about 40% of patients the diagnosis of achalasia is delayed by the reported symptoms of chest pain and heartburn simulating gastroesophageal reflux disease. As the disease progresses, inability to swallow causes malnutrition and weight loss. Squamous-cell carcinoma of the esophagus appears to develop with greater frequency in patients with long-standing achalasia than in the normal population[2].

The most sensitive tests for detecting achalasia are esophageal manometry and barium swallow videofluoroscopy. Aperistalsis and incomplete lower esophageal sphincter relaxation are the typical manometric features. Radiological abnormalities include aperistalsis, esophageal dilatation, and minimal lower esophageal sphincter opening with a bird-beak appearance. Endoscopic assessment is important to exclude the diagnosis of malignancy-induced secondary achalasia, often referred to as pseudoachalasia, before invasive therapies are implemented. Clinical features suggesting a tumor of the gastroesophageal junction are a short duration of dysphagia, a significant weight loss, and an elderly patient. Since adenocarcinoma of the cardia may present endoscopically as an infiltrating lesion with apparently normal mucosa, CT scan, endoscopic ultrasonography, and even exploratory laparoscopy should be used liberally in this subgroup of patients[3].

MANAGEMENT OF ACHALASIA

Endoscopic dilation versus surgery

Treatment of achalasia is palliative and is directed toward elimination of the outflow resistance caused by the abnormal lower esophageal sphincter function without creating excessive gastroesophageal reflux. Sustained symptomatic relief of dysphagia can be achieved by endoscopic pneumatic dilation or by surgical myotomy. Retrospective studies have shown better results with myotomy performed by an experienced surgeon, and in the only prospective randomised trial myotomy gave better long-term results compared with pneumatic dilatation[4]. A recent study of 217 patients who received a median of 4 pneumatic dilations over a 12-year period reported a long-term therapeutic success of only 50%[5]. Over the past 15 years, the advent of minimally invasive technology has made surgery a more attractive option as a first-line therapy to both patients and referring physicians[6]. It has been shown that pneumatic dilation is 72% effective versus 92% for the laparoscopic Heller myotomy. In addition, laparoscopic techniques have sharply reduced surgical morbidity[7] (Table 1).

Table 1 Pooled response rate of achalasia treatments[7]

Therapy	*n*	Weighted response rate (%)	Weighted follow-up (yr)
Botulinum toxin	149	32	1.1
Pneumatic dilation	1276	72	4.9
Heller myotomy			
Thoracotomy	1221	84	5
Laparotomy	732	85	7.6
Laparoscopy	171	92	1.2

Principles of surgical therapy

The basic goal of surgery in the treatment of achalasia is the extramucosal division of both layers of the esophageal muscolaris propria and of the oblique gastric fibers, the so-called Heller myotomy. For many years this operation has been performed through a laparotomy, often with the addition of a fundoplication, or through a left thoracotomy. The extent of the myotomy in the stomach, which in part depends on the type of surgical access, has long been a matter of controversy. Ellis, the pioneer of the transthoracic approach, advocated a limited (< 1 cm) gastric myotomy without an antireflux procedure. However, at a very late follow-up the level of symptomatic improvement markedly deteriorated over time with this approach, and the rate of excellent results (i.e., asymptomatic patients) progressively decreased from 54% at 10 years to 32% at 20 years[8]. The majority of European surgeons have instead favored the transabdominal approach which includes a longer gastric myotomy (1 to 2 cm) and a fundoplication to protect the esophagus from iatrogenic gastroesophageal reflux. Another debated issue has been the direction of the myotomy over the stomach, which may be closer to the lesser curve or to the greater curve, and, as a consequence, can divide the semicircular (clasp) fibers and the oblique (sling) fibers in distinct proportions[9].

A third major issue is the opportunity to add an antireflux repair to the myotomy. The rationale for considering a concomitant fundoplication is the assumption that a well performed surgical myotomy renders the lower esophageal sphincter incompetent. Furthermore, gastric contents propelled retrograde into an aperistaltic esophagus are not effectively cleared, thus magnifying the damage caused by postoperative reflux. The majority of surgeons worldwide presently favor a transabdominal approach and add to the myotomy a partial fundoplication, either a 180 degree anterior (Dor) or a 270 degree posterior fundoplication (Toupet). The partial fundoplication, as opposed to the 360 degree Nissen repair, does not cause significant resistance to esophageal emptying, therefore reducing the risk of postoperative dysphagia. The main advantage of an anterior Dor fundoplication is that this technique does not require mobilization of the distal esophagus and cardia, and provides a natural patch over the denuded esophageal mucosa. Overall, there has been a greater than 90% symptomatic relief and a less than 10% incidence of pH proven gastroesophageal reflux with this approach[10]. This operation should be proposed as the first therapeutic option also in patients with sigmoid-shaped esophagus, although the chances of symptomatic relief are reduced in such circumstances and in some of these individuals an esophagectomy can eventually be required.

Minimally invasive surgery for achalasia

During the early times of the minimally invasive surgical approach, both laparoscopy and thoracoscopy have been used to perform a Heller myotomy. However, it soon became clear that laparoscopy offers several inherent advantages, including superior visualization of the gastroesophageal junction, a single lumen endotracheal intubation, the ability to add an antireflux procedure, and a shorter hospital stay. In addition, laparoscopy showed a better symptomatic outcome and a lesser incidence of postoperative gastroesophageal reflux[11].

The first laparoscopic Heller procedures were performed in England and in Italy during the early 1990s[12,13]. Compared to the open abdominal approach, a technical innovation introduced in the laparoscopic era has been the use of intraoperative endoscopy as a means of precisely identifying the gastroesophageal junction, checking for the completeness of the myotomy and testing for the presence of occult perforations. During the early phase of the learning curve, a Rigiflex balloon dilator was also used to distend the cardia in order to facilitate division of all residual muscle fibers[14]. Subsequently, transillumination and air inflation provided by the endoscope were considered appropriate to assist the myotomy[15]. Further demonstration of the effectiveness of intraoperative endoscopy came from a study showing that endoscopic and laparoscopic criteria were discordant in the identification of the esophagogastric junction in 58% of the cases, the cardia being in all these cases at a more distal site with endoscopic criteria. As a consequence, based on the laparoscopic appearance, the surgeon may err by underestimating the caudal extent of the myotomy and

can perform a too short myotomy on the gastric side[16]. Although we are still convinced of the effectiveness of intraoperative endoscopy during the learning curve of this operation and during reoperative surgery, we have now discontinued this practice in our high-volume referral center where between 15 and 20 patients per year undergo Heller myotomy as a primary treatment for achalasia.

It should also be noted that a common reason for an incomplete myotomy on the gastric side is the fear of producing a mucosal injury, which typically occurs just at the esophagogastric junction. The mean reported rate of mucosal perforation is about 5%, but the frequency is largerly dependent on surgeon's experience. There are no consequences if the mucosal injury is detected intraoperatively and repaired with interrupted stitches[15].

The principle of limited surgical dissection of the cardia, already advocated by some surgeons in the open era[10], has been successfully reproduced in many centers with the minimally invasive laparoscopic approach. Dissection is limited to the anterior and lateral attachments of the phrenoesophageal membrane as this allows enough space to perform the myotomy and helps to prevent the occurrence of postoperative gastroesophageal reflux. Only in patients with an associated hiatal hernia or epiphrenic diverticulum the distal esophagus is encircled and a posterior crural repair is performed. By grasping the cardia and pulling it in a caudal direction, the myotomy is started on the distal esophagus using a L-shaped electrocautery hook until the submucosal plane is identified. The myotomy is carried out for about 6 cm on the esophagus, toward the left of the anterior vagus nerve, and up to 2 cm on the gastric side to include the oblique fibers.

Despite the minimal surgical dissection herein described, an antireflux repair added to the Heller myotomy appears to be beneficial and is nowadays supported by the majority of esophageal surgeons. The anterior Dor fundoplication is a technically simple procedure, quick and safe to perform laparoscopically. It does not require mobilization of the cardia, and places a gastric fundic patch over the myotomy site to protect the mucosa and prevent re-healing of the myotomy. Reconstruction of the angle of His is first performed by suturing the medial wall of the fundus to the left edge of the myotomy and to the left crus of the diaphragm. The Dor fundoplication is fashioned by folding the anterior fundic wall over the myotomized surface and securing the stomach to the right crus of the diaphragm and to the right edge of the myotomy with interrupted stitches. It is important to pay attention to the geometry of the fundoplication in order to avoid undue tension and to provide a uniform patch over the myotomized esophagus, but usually there is no need to divide the short gastric vessels. The Heller-Dor operation is carried out in about one hour by an expert surgeon. A gastrographin swallow study is performed the following day to check esophageal transit and to rule out leaks. A soft diet is then permitted, and the patient is usually discharged home on postoperative d 2 or 3.

Studies including large number of patients have shown that an extramucosal myotomy of the esophagus and cardia combined with an anterior fundoplication can be performed safely and effectively through laparoscopy, with clinical and functional results similar to that obtained with the open transabdominal approach and relief of dysphagia in more than 90% of patients[17-22]. Although previous endoscopic treatments, such as balloon dilatation or intrasphincteric botulinum toxin injection, may cause submucosal scarring at the esophagogastric junction resulting in a more difficult surgical procedure and an increased operative morbility, no statistically significant differences as far as concern the clinical outcome have been reported[15].

Table 2 Twenty-four h pH data of patients submitted to Heller myotomy and Heller myotomy plus Dor fundoplication in a randomized study[24]

Parameter	Heller (n = 18)	Heller-Dor (n = 21)	P value
Time (%) pH < 4 upright	8.1 + 10.4	0.8 + 1.1	0.015
Time (%) pH < 4 supine	9.1 + 18.3	2.0 + 6.9	0.002
No. episodes pH < 4	113 + 128	25 + 6.9	0.001
No. episodes pH < 4 for ⩽ 5 min	3.4 + 4.7	0.5 + 1.6	0.001

Radiologic and manometric studies after the laparoscopic Heller-Dor operation have shown a significant decrease of the internal diameter of the thoracic esophagus and of the resting lower esophageal sphincter pressures[19]. In a recent report, at a minimum follow-up of 6 years after the operation, about 82% of patients were satisfied with the treatment and were able to eat normally; more than a half of the symptomatic recurrences occurred during the first year of follow-up and were effectively treated with pneumatic dilations. Nine (12.7%) patients either had abnormal acid exposure on postoperative 24-h pH study or were on treatment with proton-pump inhibitors for reflux symptoms; however, in none of these individuals did endoscopy reveal more than grade A esophagitis[23].

An important preoperative factor affecting the outcome of the laparoscopic operation is the magnitude of the resting pressure of the lower esophageal sphincter. On a multivariate analysis, only a high resting pressure prior to surgery was a predictor of resolution of dysphagia. Interestingly, all patients with a preoperative sphincter pressure greater than 36 mmHg had their dysphagia resolved after surgery[24].

The issue of whether an antireflux procedure should be added to the Heller myotomy has long been controversial and supported only by personal feelings and retrospective studies. Recently, a randomised double-blind clinical trial comparing the outcome of myotomy plus Dor fundoplication versus myotomy alone has shown that the former operation is superior in terms of reflux control (Table 2). The addition of a Dor fundoplication reduces the risk of pathologic gastroesophageal reflux by 9-fold as tested by 24-hour esophageal pH monitoring. Even the median esophageal acid exposure was lower in the Heller-Dor group, indicating that the few unfortunate individuals in whom reflux might occur are easily managed with

medical therapy[25].

The predominant mechanism of failure after the Heller operation is an incomplete distal myotomy. In such circumstances, pneumatic dilatation can represent a reasonable therapeutic option when dysphagia is mild. In patients with major symptoms, revisional laparoscopic surgery with repeat myotomy and fundoplication is feasible and effective with a low morbidity rate[26]. Intraoperative endoscopy is helpful as a guide during dissection to identify the cleavage plans and to clearly recognize the gastroesophageal junction. When a properly performed myotomy has failed in a patient with sigmoid esophagus, and the redundant supradiaphragmatic esophageal loop still interferes with emptying, a transthoracic or a transhiatal esophagectomy is the treatment of choice[27].

It has recently been suggested that a laparoscopic myotomy extended for 3 cm on the gastric side can result in better dysphagia scores and in fewer additional procedures to treat recurrent dysphagia, provided that a Toupet fundoplication is added to the procedure[28]. However, this improved outcome should be balanced against the risk of severe gastroesophageal reflux disease induced by the longer gastric myotomy and the necessity to fully mobilize the cardia to perform a posterior fundoplication. Interestingly, a very long-term study of 67 patients who underwent an open Heller-Dor operation and were followed for more than 20 years has shown a progressive clinical deterioration of results and a 22.4% failure rate due to an increased esophageal acid exposure and development of Barrett's esophagus[29], suggesting once again that surgical therapy for achalasia involves a very delicate balance between relief of outflow obstruction and destruction of the antireflux barrier.

Robot-assisted Heller operation has been performed with satisfactory outcomes and no mucosal perforation[30]. Operative times were similar to those of standard laparoscopic operation after the first 30 cases in a multicenter study[31]. No data from randomised clinical trials have been reported yet. At present time, despite the advantage of the three-dimensional view, elimination of tremor, and 360 degree of freedom of movement of the robotic arms, the role of this technology in the management of esophageal achalasia remains to be determined.

CONCLUSIONS

The management of achalasia has changed significantly over the past 15 years. Minimally invasive surgery has influenced the management of esophageal disease more than any other gastrointestinal disorder and in a manner similar as in the therapy of cholelithiasis. In several institutions around the world, the laparoscopic surgical myotomy has replaced pneumatic dilation as the initial therapy of choice for achalasia. Current evidence from the literature suggests that it is conceivable to extend the Heller myotomy on the gastric side for about 2 cm to encompass the oblique fibers without fear of inducing significant gastroesophageal reflux, provided that a minimal antero-lateral dissection of the phrenoesophageal membrane is performed and a Dor fundoplication is routinely added to the myotomy.

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EDITORIAL

New methods for the management of gastric varices

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Abstract

Bleeding from gastric varices has been successfully treated by endoscopic modalities. Once the bleeding from the gastric varices is stabilized, endoscopic treatment and/or interventional radiology should be performed to eradicate varices completely. Partial splenic artery embolization is a supplemental treatment to prolong the obliteration of the veins feeding and/or draining the varices. The overall incidence of bleeding from gastric varices is lower than that from esophageal varices. No studies to date have definitively characterized the causal factors behind bleeding from gastric varices. The initial episodes of bleeding from esophageal varices or gastric varices without prior treatment may be at least partly triggered by a violation of the mucosal barrier overlying varices. This is especially likely in the case of varices of the fundus. In view of the high rate of hemostasis achieved among bleeding gastric varices, treatment should be administered in selective cases. Among untreated cases, steps to prevent gastric mucosal injury confer very important protection against gastric variceal bleeding.

Key words: Gastric varices; Esophageal varices; Bleeding

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INTRODUCTION

Bleeding from esophageal varices (EVs) or gastric varices (GVs) is a catastrophic complication of chronic liver disease. Bleeding from GVs is generally thought to be more severe than bleeding from EVs[1], but it occurs less frequently[2-4]. Though many recent developments have improved the outcome of treatments for GVs, no consensus has been reached on the optimum treatment. In this paper we review the pathomorphology, hemodynamics, risk factors for bleeding, and treatments for GVs. In the esophagogastric varices grading system of the Japan Society for Portal Hypertension[5], the varices are evaluated based on color (white [Cw], and blue [Cb]), form (small and straight [F1], nodular [F2], and large or coiled [F3]), and the red color sign (RC0-3). GVs are divided into cardiac varices (Lg-c), fundal varices (Lg-f), and varices involved both the cardia and fundus (Lg-cf). In this review, GVs are divided into two categories and described accordingly: Lg-c (cardiac varices: CVs) and Lg-cf or Lg-f (fundal varices: FVs).

PATHOMORPHOLOGY OF GVs

Arakawa *et al*[6] reported that CVs are supplied by the left gastric vein (cardiac branch), a vessel which enters the stomach wall in the cardia at a point 2 to 3 cm from the esophagogastric junction and diverges into a profusion of branches running throughout the cardia. Some of these veins become markedly dilated, acquiring the features of varices. Most veins in the cardia diverge into parallel veins from the esophagogastric junction as the flow becomes hepatofugal. However, Others will dilate, wind through the submucosa, and directly join EVs. Histologically, nearly the entire cross-section of the wall is the varix itself. The varices are covered by thinning layers of serosa and mucosa through which they can ultimately be seen.

The angio-architecture of a FV is quite different from that of a CV. Most FVs are supplied by the short gastric vein, though in some cases the blood is fed from the posterior or left gastric vein. Thus, the vascular anatomy of a FV is something like a splenorenal shunt running through the stomach wall. Bleeding from an EV most commonly occurs in the "critical area" 3 cm proximal to the esophagocardiac junction. Fine longitudinal veins in the lamina propria originate at the esophagocardiac junction and run in the lamina propria toward this critical area. EVs consist of multiple dilated veins. Those that rupture are usually located in the lamina propria[7].

In the stomach, unlike its counterpart the esophagus a large winding vein runs through the submucosa without causing varicose veins to pile up. The ruptures in CVs occur in the submucosa, where they disrupt the muscularis mucosae and lamina propria mucosae. The mucosal layer covering a FV is somewhat thicker than that covering an EV. The difference between a CV and a FV lies in the

caliber of the varicose vein and the degree of vascular anastomosis. Most FVs are supplied *via* the short gastric vein, though some are fed by the posterior or left gastric vein. Anastomosis of FVs is generally uncommon. The varices within the wall penetrate the muscle layer and wind through the submucosal layer, where they displace and attenuate the muscularis mucosae and propria mucosae. The varicose veins protrude into the gastric lumen.

The lamina muscularis mucosa in the esophageal mucosa is loose, and the venous pressure in the submucosa is transmitted through communicating branches to the veins in the lamina propria. The lamina muscularis mucosa in the gastric mucosa, on the other hand, is tightly integrated with the lamina propria[8].

The red color sign is an elevated red area which has proven to be important in portending variceal bleeding. The histological manifestation is a thinning of the epithelial layer The North Italian Endoscopic Club for the Study and Treatment of Esophageal varices[9] published a report establishing that the red color sign on EVs is predictive of bleeding. It remains unclear whether the endoscopic red color sign in the stomach has the same significance as the red color sign in the esophagus. In the latter case does it denote a thinning of the epithelial layer. The varix in the submucosa of the stomach is covered by the muscularis mucosae and propria mucosae. This generally confers an appearance different from that typical of the thinning epithelial layer of the esophagus[6].

HEMODYNAMICS OF GVs

The portal hemodynamics of GVs should be evaluated in all patients with these varices to determine the most appropriate treatment. CVs are supplied by the left gastric vein (cardiac branch), a vessel which enters the stomach wall in the cardia at a point 2 to 3 cm from the esophagogastric junction and diverges into a profusion of branches running throughout the cardia. The main veins feeding the FVs are the left gastric vein (51%), posterior gastric vein (30%), and short gastric vein (69%). The principal drainage veins for the FVs are the gastro-renal shunt (87%) and gastric-inferior phrenic vein shunt (16%), though about 1% of FVs reported to communicate with neither[10]. FVs are more frequently supplied by the short and posterior gastric veins than CVs. Concomitant collaterals such as EVs, para-esophageal veins, and para-umbilical veins are additionally observed in nearly 60% of FVs.

RISK FACTOR FOR BLEEDING FROM GVs

The incidence of variceal bleeding in patients who have never received treatment for EVs has been reported to range from 16 to 75.6%[11,12]. The incidence of bleeding from GVs stands at 25%[2], while cumulative bleeding rates from FVs at 1, 3, and 5 years have been estimated at 16%, 36%, and 44%, respectively[13]. Thus, the overall incidence of bleeding from GVs is lower than that from EVs[2]. In an earlier study on the natural course of GVs in 52 patients, our group treated bleeding from GVs in 4 patients over a mean follow-up period of 41 mo. Hemorrhage was successfully halted in all 4 of these patients. The cumulative bleeding rates at 1, 3, and 5 years were 3.8%, 9.4%, and 9.4%, respectively. Three of the 4 patients were free of erosive gastritis and gastric ulcer at the time of entry into the study, though ulcers or erosions were found at the bleeding points of the GVs in all 4 when the varices ruptured. There were no significant differences in patient characteristics with ruptured *versus* non-ruptured GVs when the patients entered the study[4].

The endoscopic risk factors for bleeding from EVs include the presence of raised red markings, cherry-red spots, blue color, and large size[14]. The risk factors for bleeding from GVs have yet to be characterized. In another study, our group examined 70 cirrhotic patients with first-time bleeding from EVs or GVs without prior treatment[15]. The red color sign was more common in EVs than in CVs or FVs ($P < 0.0001$). Mucosal erosion over the varices at the site of bleeding was more common in CVs ($P < 0.0005$) and FVs ($P < 0.0001$) than in EVs. An ulcer at the bleeding point was more common in CVs ($P < 0.01$) and FVs ($P < 0.0001$) than in EVs. Gastric ulcer was more common in CVs ($P < 0.05$) and FVs ($P < 0.001$) than in EVs. Erosive gastritis was more common in FVs ($P < 0.02$) than in EVs. The red color sign, a strong risk factor for the ruptures frequently encountered in EVs, was completely absent in the FVs. All of the CVs manifesting the red color sign communicated with EVs which manifested the red color sign themselves. This might have been due to the pronounced thickness of the mucosal layer overlying the FVs. FVs are usually two or three times larger than EVs and drain directly into an extremely dilated left gastric or posterior gastric vein[16]. The volume of blood flow through a FV therefore usually exceeds that through an EV. Gastric ulcers that develop over GVs represent violation of the protective layer of gastric mucosa. Violation of the mucosal barrier overlying GVs place patients at risk of massive bleeding, especially when FVs are involved. Violations of this type could be an important precondition leading to variceal hemorrhage.

TREATMENT OF GVs

Treatment modalities for GVs include balloon tamponade, endoscopic treatment, embolization, and surgery.

Balloon tamponade

Balloon tamponade with the Sengstaken-Blakemore or the Linton-Nachlas tube is an emergent procedure for active hemorrhaging from GVs. The procedure is effective in the short term, but permanent hemostasis is obtained in fewer than 50% of cases[17,18].

Endoscopic treatment

Two endoscopic treatment modalities are used for the treatment of esophagogastric varices: endoscopic injection sclerotherapy (EIS) and endoscopic variceal ligation (EVL)[19-26]. EIS can be accomplished by either intravariceal EIS or extravariceal EIS[21-23,26]. In the treatment of EVs, intravariceal EIS obliterates both the interconnecting perforating veins and the veins feeding the EVs. Most veins in the cardia become parallel veins from the

esophagogastric junction at the point at which the flow becomes hepatofugal. Nearby, however, a number of dilated winding cardiac veins run through the submucosa and directly join the EVs. This makes it possible to treat most CVs concomitantly with EVs when correcting the latter by intravariceal EIS.

EIS and EVL are both effective for the treatment of bleeding from EVs and CVs. EIS has been less successful in the treatment of bleeding from FVs, however. When used with 1% polidocanol, 5% ethanolamine oleate iopamidol (EOI), or thrombin for this purpose, EIS has a high rate of operative mortality[27-29]. Fortunately, the rate of initial hemostasis has been significantly improved since the introduction of *N*-butyl-2-cyanoacrylate (Histoacryl) as the sclerosant in EIS[30,31]. We should note, however, that bleeding from the GVs injection site and rebleeding from the rupture point have been reported in patients receiving EIS[2,29].

While EVL is generally safe and effective for the treatment of CVs and FVs[32], it sometimes causes deep or extensive ulcers and increases the risk of ensuing ulcer hemorrhage or secondary bleeding[33]. FVs are usually twice or three times larger than EVs and are directly connected to an extremely dilated left gastric or posterior gastric vein[16]. The volume of blood flow through an FV therefore usually exceeds that through an EV[34]. A mucosal injury remains on the varices after endoscopic treatment. If the blood flow in the varices cannot be stopped completely, bleeding may recur at the site of this mucosal injury. This underlines the importance of ensuring the complete obliteration of the blood flow when treating FVs endoscopically. It may be dangerous to treat FVs by EVL alone.

GVs have also been treated by a combined endoscopic method using a detachable snare and simultaneous EIS and O-ring ligation[35]. This technique is not yet in widespread use, however. Our group published a report on the treatment of ruptured GVs by EIS with Histoacryl followed by O-ring ligation (endoscopic scleroligation: EISL)[24]. EISL was developed as a treatment modality for EVs to prevent bleeding from the injection site during needle removal[21,36]. When treating GVs by EIS with Histoacryl, the immediate freezing of the Histoacryl around the needle hinders the removal of the needle after the injection. In some cases, bleeding from the GV injection site or rebleeding from the rupture point also occurs[2,29]. Our group used the EISL procedure to treat ruptured GVs with punctures near the rupture points by simultaneous ligations of the injection sites and rupture points. EISL effectively stopped the bleeding from the GVs, enabled swift and easy needle removal, and successfully eliminated both bleeding from the injection site and rebleeding from the rupture point. An O-ring was placed at the point of the EISL injection with Histoacryl and left in position for a long time. As of this writing, EISL with Histoacryl is considered the most promising treatment for hemorrhaging GVs.

Interventional radiology (IVR)

The portal hemodynamics of GVs, the main feeding veins from the portal system, and the main drainage veins into the vena cava should be determined in all patients with the GV. Angiography can determine the hemodynamics of the GV simultaneously during treatment by embolization.

Transportal obliteration: Two methods have been used to obliterate the feeding veins of GVs: percutaneous transhepatic obliteration and trans-ileocolic vein obliteration. The catheter is inserted directly into the portal vein, the portal circulation is visualized by portography, a balloon catheter is inserted selectively into the inflow site of the feeding veins for the varices, the balloon is inflated, and a test dose of a contrast medium is injected to determine the optimal volume of sclerosant fluid. Five percent EOI and/or 500 g/L glucose is injected to obliterate the feeding vein, then steel coils are used to complete the obliteration[37]. The procedure is quite effective, though only transportal obliteration is sometimes incomplete, especially in FVs.

Balloon-occluded retrograde transvenous obliteration (BRTO): BRTO is a notable IVR procedure developed specially for the treatment of FVs. The technique is performed by inserting a balloon catheter into the outflow shunt (gastric-renal shunt or gastric-inferior phrenic vein shunt) *via* the femoral or internal jugular vein. Any existing collateral veins are treated with coils, absolute ethanol, or a small amount of 5% EOI. The balloon is inflated and a test dose of contrast medium is injected to determine the optimal volume of the sclerosant solution. Five percent EOI is slowly injected through the catheter until the shunt is filled with the sclerosant fluid. The catheter is removed after 24 h of balloon occlusion[38-40]. A remarkably high rate of FV eradication or reduction in FV size can be expected if the BRTO procedure is technically successful. Indeed, long-term eradication of treated FVs without recurrence is achieved in most patients[38,41]. Kanagawa *et al*[38] confirmed eradication of FVs in 97% of 32 patients treated by this procedure, and no FVs recurred in any of those patients within an average follow-up period of 14 mo. In earlier reports, the eradication rate of FVs exceeded 89% and the recurrence rate was less then 7%. In light of the minimal invasiveness and high safety of the procedure, BRTO is applicable not only for elective cases, but also for emergency cases with FVs.

FV treatment by BRTO has two significant effects, namely, eradication of the FVs themselves and obliteration of the unified portal-systemic shunt. Thus, most of the benefits and adverse effects of BRTO are related to obliteration of the unified portal-systemic shunt. Benefits such as decreased blood ammonia levels and improved porto-systemic encephalopathy are sometimes observed. Possible adverse effects include transient ascites, increases of ascites, pleural effusion, and the appearance of EVs manifesting the red color sign. These adverse effects may be due to elevation of the portal pressure in reaction to the occlusion of the portal-systemic shunt.

Partial splenic artery embolization (PSE): The femoral artery approach is used for super-selective catheterization of the splenic artery. The catheter tip is placed as distally as possible in either the hilus of the spleen or in an intrasplenic artery. Embolization is achieved by injecting 2-mm cubes of gelatin sponge suspended in a saline solution containing antibiotics[42,43]. PSE has been

performed to treat hypersplenism, EVs, GVs, portal hypertensive gastropathy, pancreatic carcinoma, and portosystemic encephalopathy[37,43-53]. Our group evaluated PSE in a long-term study of 26 patients with hepatic cirrhosis alongside 26 patients who did not undergo the PSE procedure[42]. The red blood cell counts of the PSE (+) group increased significantly by 6 mo after the procedure and remained increased for up to 7.5 years. The platelet counts peaked only 2 wk after PSE and gradually fell thereafter. Even so, the platelet counts remained significantly higher than the pre-PSE level for up to 8 years. No significant changes were observed in the aspartate aminotransferase and alanine aminotransferase activities in serum during the follow-up. Cholinesterase activity was increased significantly by 6 mo after PSE and remained increased for more than 7 years. The serum albumin concentration increased significantly from 6 mo after PSE and the level remained significantly increased for 6 years. Survival did not differ between the PSE (+) and PSE (-) groups. PSE, a non-surgical treatment, can benefit patients with cirrhosis by improving the capacity of hepatic protein synthesis and conferring protection against hemorrhage due to thrombocytopenia.

Combination modalities with IVR: Our group also reported the long-term results of PSE as supplemental treatment for portal-systemic encephalopathy. We divided 25 patients with portal-systemic encephalopathy due to portal-systemic shunts into two groups, one treated by transportal obliteration and/or BRTO of portal-systemic shunt, followed by PSE (PSE (+) group; $n = 14$), the other treated by transportal obliteration and/or BRTO of the portal-systemic shunt without PSE (PSE (-) group; $n = 11$). The serum ammonia levels and grades of encephalopathy were lower in the PSE (+) group than in the PSE (-) group at 6, 9, 12, and 24 mo after treatment. Obliteration of the portal-systemic shunt raised the portal venous pressure in every case. As all of the patients had cirrhosis, the portal-systemic shunt drainage reduced portal hypertension and the obliteration of the portal-systemic shunt led to portal congestion and increased portal venous pressure. Our study thus confirmed the benefits of obliteration of the portal-systemic shunt by PSE in patients with portal-systemic encephalopathy[43].

PSE is performed incrementally during the monitoring of the portal pressure in order to reduce the portal venous pressure to the level measured before obliteration of the veins feeding and/or draining the GVs[22,42,43,49,54]. PSE is a supplemental modality to prolong the effect of obliteration of the veins feeding and/or draining the GVs.

Combination of endoscopic treatment and IVR

Treatment of GVs solely by endoscopic modalities or by IVR is occasionally incomplete. Our group previously reported that combined treatments with IVRs and endoscopic modalities had significant impacts on long-term rebleeding and retreatment rates in patients with EVs or GVs[37,48,50,51]. In elective cases, complete GV treatment should be administered in order to prevent rebleeding with greater assurance.

Surgery

A number of surgical procedures have been developed to manage esophagogastric varices. These can be classified as shunting and nonshunting procedures. The goal of shunting is to reduce the incidence of variceal bleeding by lowering the pressure in the portal system using a portal-systemic shunt. While the standard portocaval shunt effectively reduces the incidence of variceal bleeding, impairment of the hepatic protein metabolism in patients undergoing the procedure frequently leads to the development of hepatic encephalopathy due to hyperammonemia[55-57]. The distal splenorenal shunt (DSRS) was developed by Warren *et al*[58] in 1967 as a way to preserve portal blood flow through the liver while lowering variceal pressure. The hope, in developing this approach, was to prevent both bleeding and hyperammonemia. While DSRS effectively prevents rebleeding, patients who undergo DSRS still can develop hyperammonemia. Our group responded by designing a DSRS with a splenopancreatic disconnection and gastric transection, modifications to prevent the loss of shunt selectivity. This modified DSRS has been proved to reduce the incidence of postoperative hyperammonemia[59].

As an alternative to shunting, Hassab[60] and Sugiura *et al*[61] developed a method of gastro-esophageal decongestion and splenectomy for the treatment of varices. The Hassab operation devascularizes the distal esophagus and proximal stomach. Splenectomy, selective vagotomy, and pyloroplasty can be performed concomitantly with the procedure. Sugiura *et al*[61] developed a method of esophageal transection for patients with GVs and EVs. Sugiura's method is performed concomitantly with the Hassab operation to divide and reanastomose the distal esophagus in order to disrupt the blood supply to the EVs. While both procedures may solve the problem of hepatic encephalopathy, varices are likely to recur earlier than they are in patients undergoing DSRS[62].

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REVIEW

Complementary analysis of microsatellite tumor profile and mismatch repair defects in colorectal carcinomas

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Abstract

Microsatellite instability (MSI) is a prognostic factor and a marker of deficient mismatch repair (MMR) in colorectal adenocarcinomas (CRC). However, a proper application of this marker requires understanding the following: (1) The MSI concept: The PCR approach must amplify the correct locus and accurately identify the microsatellite pattern in the patient's normal tissue. MSI is demonstrated when the length of DNA sequences in a tumor differs from that of nontumor tissue. Any anomalous expansion or reduction of tandem repeats results in extra-bands normally located in the expected size range (100 bp, above or below the expected product), differ from the germline pattern by some multiple of the repeating unit, and must show appropriate stutter. (2) MSI mechanisms: MMR gene inactivation (by either mutation or protein down-regulation as frequently present in deep CRC compartments) leads to mutation accumulation in a cell with every cellular division, resulting in malignant transformation. These mechanisms can express tumor progression and result in a decreased prevalence of aneuploid cells and loss of the physiologic cell kinetic correlations in the deep CRC compartments. MSI molecular mechanisms are not necessarily independent from chromosomal instability and may coexist in a given CRC. (3) Because of intratumoural heterogeneity, at least two samples from each CRC should be screened, preferably from the superficial (tumor cells above the muscularis propria) and deep (tumor cells infiltrating the muscularis propria) CRC compartments to cover the topographic tumor heterogeneity. (4) Pathologists play a critical role in identifying microsatellite-unstable CRC, such as occur in young patients with synchronous or metachronous tumors or with tumors showing classic histologic features. In these cases, MSI testing and/or MMR immunohistochemistry are advisable, along with gene sequencing and genetic counseling if appropriate. MSI is an excellent functional and prognostically useful marker, whereas MMR immunohistochemistry can guide gene sequencing.

Key words: Colon carcinoma; Microsatellites; Mismatch Repair; Hereditary non-polyposis colon cancer

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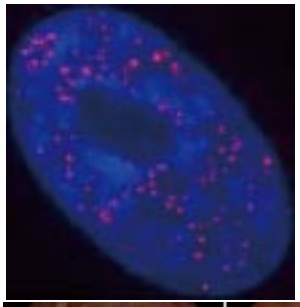
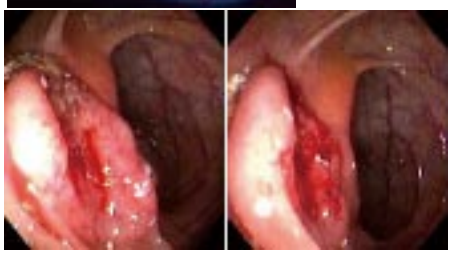
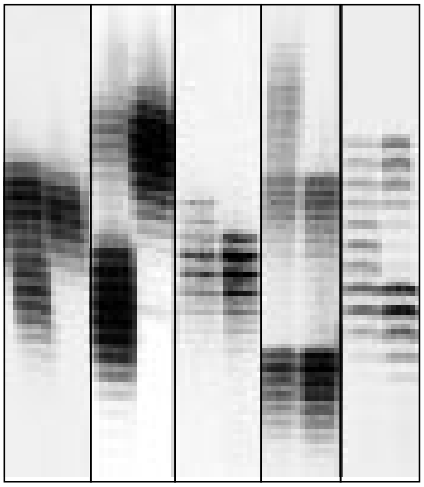
INTRODUCTION

Colorectal carcinoma (CRC) is generally classified into three categories, based on increasing hereditary influence and cancer risk[1,2]: sporadic CRC (approximately 60% of cases and comprises patients with no notable family history and, by definition, with no identifiable inherited gene mutation that accelerates cancer development), familial CRC (approximately 30% of cases and refers to patients who have at least one blood relative with CRC or an adenoma, but with no specific germline mutation or clear pattern of inheritance), and hereditary CRC syndromes (approximately 10% of cases, which result from inheritance of a single gene mutation in highly penetrant cancer susceptibility genes). Although the last group has the lowest frequency, it has elucidated molecular mechanisms of carcinogenesis applicable to sporadic CRC[3].

The microsatellite profile of sporadic CRC is a prognostically useful marker[4-7]. Microsatellites are repeating DNA sequences of unknown function that are found throughout the genome[8]. Microsatellite instability (MSI) is demonstrated when the length of DNA sequences in tumor and nontumor tissues is different and MSI has been identified in a wide variety of human tumors, due to defects in one of the DNA mismatch repair (MMR) genes, especially *MLH1* or *MSH2*[1,9]. However, MSI presence alone does not establish a diagnosis of hereditary nonpolyposis colon cancer (HNPCC) because MSI has also been identified in 10%-30% of sporadic CRC. Certain histological features also correlate with the presence of







MSI CRC	Feature	MSS CRC
94%	Right side	34%
59%	Size > 6 cm	29%
53%	Poorly differentiated	7%
35%	Mucinous	7%
47%	Lymph infiltrates	10%

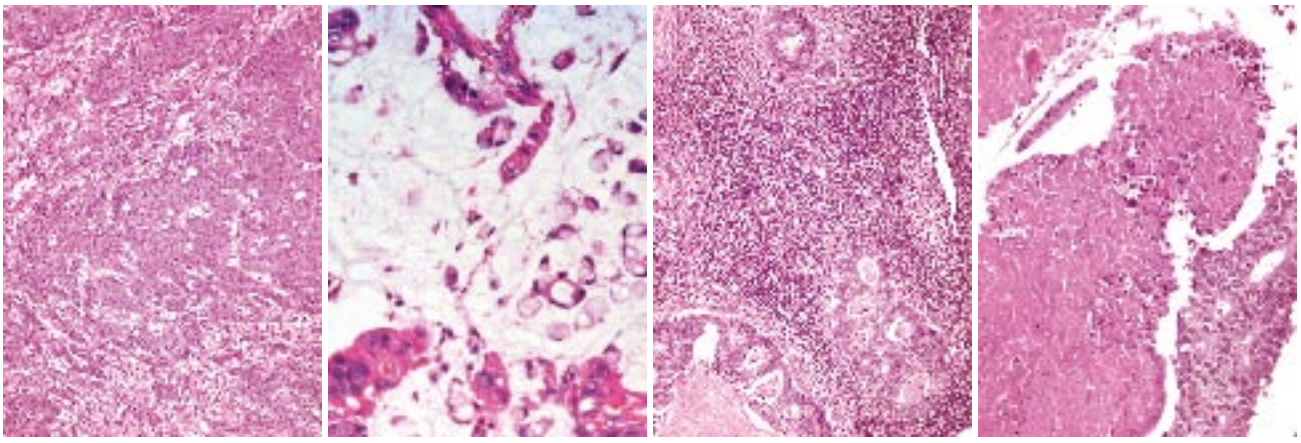


Figure 1 Microsatellite unstable CRCs are normally located proximal to the splenic flexure and reveal non-polypoid pattern. MSI can be demonstrated by fluorescence in-situ hybridization (FISH) or molecular techniques. Histopathologically, these tumors show solid growth, mucinous differentiation, prominent lymphocytic infiltrate, and no dirty necrosis[10].

MSI in sporadic CRC (Figure 1)[5,10-13], which can be key elements in the design of more effective therapeutic protocols[12,13].

Both basic and clinical implications of MSI and MMR defects need to be considered in an appropriate context, which requires clarifying the definition of MSI, the biological consequences of tumor MSI, interference of intratumor heterogeneity on MSI detection, differences in clinical testing for MSI and for MMR defects, and MSI prognostic and therapeutic implications.

MSI DEFINITION AND CLINICAL TESTING FOR MSI

Any useful application of prognostic factors requires a reliable definition of the factor. Microsatellites belong to the family of highly polymorphic and repetitive non-coding DNA sequences that, although widely distributed in the human genome, are not uniformly spaced (underrepresented in subtelomeric chromosome regions). Microsatellites are useful molecular markers due to their

ubiquity, PCR typability (except for $(dA)_n$ multimers, whose size polymorphisms are difficult to type), Mendelian co-dominant inheritance, and extreme polymorphism[8], but their origin and function are not clear[14]. They have been demonstrated to be very useful in cell lineage delineation, positional cloning, and several applications in forensic medicine[15,16]. Microsatellite instability (MSI) is demonstrated when the lengths of DNA sequences in a tumor differ from those of nontumor tissue. MSI has been identified in a wide variety of human tumors.

Currently, tumor MSI analyses require molecular tests and the application of strict criteria. MSI can be defined as a change in any DNA sequence length due to either insertion or deletion of repeating units in a microsatellite within a tumor when compared to normal tissue[17,18]. The tests must be run with appropriate controls (known positive and negative controls along with the patient's normal tissue)[8,19], which are extremely important due to the non-exceptional presence of extra-bands. The PCR approach must amplify the correct locus and accurately identify the microsatellite pattern in the patient's normal tissue. Any anomalous expansion or reduction of tandem repeats due to MSI results in extra-bands. True extra-bands expressing tumor MSI are normally located in the expected size range (usually about 100 bp), are above or below the expected PCR product, differ from the germline pattern by some multiple of the repeating unit (e.g. delta 6 bases for dinucleotides), must show appropriate stutter (e.g. -2, -4 for dinucleotides), and are not present in the normal control. These tests should be carefully analyzed considering the following: (1) Sample homogeneity/heterogeneity can vary. Very small samples (even single cells) have been used in genetic analyses to avoid normal cell contamination. However, the lower the number of cells the higher the probability of technically-related abnormal results[20], which can be partially resolved with appropriate methods. The high incidence of PCR artifacts using microdissected samples is related to the small concentration of target DNA, fixation induced changes of DNA, and conditions in the amplification of repetitive sequences (especially for those CG-rich sequences) favouring misannealing and hairpin formation. Appropriate modifications to avoid the above conditions will significantly improve the reproducibility of LOH and MSI tests in microdissected samples[21]. (2) Appropriate controls are necessary for every step of the molecular tests to avoid false results. Sufficient levels of amplification with all markers should be obtained to detect low amounts of shifted microsatellites. (3) PCR bias against one allele (especially the larger one in a pair) can result in preferential amplification of the other allele (usually the smaller in a pair), which is the so-called artifactual allele dropout[22,23]. An appropriate extraction method, providing DNA of quality[24], and PCR designs including both long denaturation and extension in the first three cycles and 7-deaza-dGTP in the amplification mixture to improve the amplification of CG-rich DNA regions, will be reasonably helpful in avoiding that bias[8,19,21,23,25]. (4) The number of polymorphic DNA regions agreed to at the NCI consensus conference includes a primary panel of at least 2 mononucleotide and 3 dinucleotide microsatellites, along with 19 alternate loci (both mono- and dinucleotides)[26]. The choice of microsatellite markers is important in MSI testing, with the examination of mononucleotide repeats being sufficient for detection of MMR deficient tumors, whereas instability only in dinucleotides is characteristic of MSI-L/MMR-positive tumors[27]. Depending on the number of abnormal loci from the total analyzed, the cases are classified into MSI-high ($\geq$ 30%-40% of abnormal loci), MSI-low ($<$ 30%-40% of abnormal loci), and MSS (no abnormal loci).

Which patients should be tested? The neoplasm histological features closely correlate with MSI and should be the key elements used to select sporadic CRC for MSI investigation[5,10-13]. The sets of criteria for the clinical diagnosis of HNPCC appear under Clinical Testing for MMR defects. The implications of these analyses in sporadic and HNPCC carcinomas are compared in Table 1.

Table 1 False negative in antigen positive neoplasms,comparative features of microsatellite unstable sporadic adenocarcinoma and hnpcc colon carcinomas

	MSI-H sporadic adenocarcinomas	HNPCC adenocarcinomas
Patient age	Older	Younger
Number of tumors	Single	Single/Multiple (synchronic/ metachronic)
Colonic distribution	Right colon	Right colon
Histological clues	Poorly differentiated, medullary type Crohn-like inflammation	Poorly differentiated, medullary type Crohn-like inflammation
Mechanism of MMR deficiency	MLH1 promoter hypermethylation	Inactivating germline mutation of MMR proteins
Tumor prognosis	Better than MSI-L/ MSS sporadic adenoca	Better than MSI-L/ MSS sporadic adenoca

BIOLOGICAL CONSEQUENCES OF TUMOR MSI

Microsatellite-unstable CRC are biologically different and have a better survival rate than sporadic CRC when matched for cancer stage[28-30]. The development of proximal and distal CRC involves partly different mechanisms associated with the MSI and the chromosomal instability (CIN) pathways[31].

These two pathways are not always independent and some CRCs show a significant degree of overlap between these two mechanisms[32]. In one study, 35% of CRC were microsatellite-unstable (21% MSI-low and 14% were MSI-high) and 51% of CRC had at least one LOH event, with the most frequent chromosomal losses observed on 18q (72.5%)[32]. A significant degree of overlap between MSI and CIN pathways has been reported in that series: 6.5% of CRC with LOH were also MSI-high, and 23.3% of MSI-high CRC also had one or more LOH events. These data suggest that molecular mechanisms of genomic instability are not necessarily independent and may not

be fully defined by either the MSI or CIN pathways. In addition, a subgroup of CRCs showed no evidence of alterations in either of these two pathways of genomic instability (37.8% of microsatellite-stable CRCs had no LOH events identified)[32], a situation similar to that reported in muscle-invasive transitional cell carcinomas of the bladder[25].

MMR proteins normally identify and correct mismatched DNA sequences that can occur during DNA replication. An inactivating mutation in any of these genes leads to mutation accumulation in a cell with every cellular division, resulting in malignant transformation[6,8,33-36]. Tumor progression in the deep compartments may be the result of MMR protein down-regulation, which would contribute to the following: (1) There is a decreased prevalence of aneuploid cell lines and K-RAS and B-RAF mutations detected in microsatellite-unstable CRC and in the deep compartments of sporadic CRC[7,29,37,38]. Microsatellite-unstable CRCs tend to be diploid[37,39], and to have lower DNA indices[39]. Supporting these findings, the MMR protein down-regulation observed in the deep compartments of sporadic CRC has shown correlation with increased frequency of diploid DNA content[40,41]. (2) Differential cell kinetics (proliferation and apoptosis) has been identified in superficial and deep compartments (above muscularis propria vs. muscularis propria) of sporadic CRC, which has demonstrated a close correlation with MMR protein expression (Figure 2)[41,42]. Physiologic correlations between MMR protein expression and kinetic variables (mitotic figures, Ki-67 expression, ISEL index) were preserved in the superficial compartment only. In addition, G_2 + M phase fraction correlated with hMLH1 expression only in superficial compartments and hMSH2 expression only in deep compartments. Both the high cellular turnover and the maintained cell kinetic balance suggest that superficial compartments of sporadic CRC are expansile. In the deep compartments, the expression of MMR proteins is inefficient (not correlated with G_2 + M phase fraction) and is dissociated (only one gene product correlates with G_2 + M), which would eventually result in mutation accumulation and progression[41].

INTRATUMOR HETEROGENEITY AND MICROSATELLITE ANALYSIS

Tumor cell heterogeneity is linked to genetic instability and biologic progression. This problem must be studied by including several tumor samples of sufficient size from each tumor.

The sample size is an important parameter. Microdissection techniques allow selectively picking up very small samples, which can show false cellular homogeneity, based on the loss of heterozygosity or allelic imbalance. If the tumor cell populations selected for molecular analysis are taken before they become a biologically prominent component (with kinetic or invasive advantages), the results might be confusing and clinically non-relevant. This would be a case of tumor microheterogeneity, which tends to give disparate results with meanings essentially unknown. Except for intraepithelial proliferation, all microdissected cell samples provide target cell-rich samples with varying degrees of host cell contamination (including stromal, inflammatory, and endothelial cells). Therefore, multiple samples from the same case should always be studied and assays performed in duplicate before accepting the results as relevant.

The intratumor heterogeneity can result in discordant results for a given marker depending on the sample origin. The comparison of MMR protein expression and PCR-based MSI studies has revealed discordant results in 8% of right-sided sporadic CRC and complete concordance after performing further analyses on other tumor areas[43]. Because of this intratumor heterogeneity, at least two samples from each CRC should be screened, although no systematic approach has been used to address this topic in sporadic CRC. Microsatellite analysis in muscle-invasive transitional cell carcinomas of the bladder have revealed topographic heterogeneity in 32% of cases, showing that the deep compartment had more microsatellite abnormalities (20%)[25]. We have found significant differences between superficial (tumor cells above the muscularis propria) and the deep (tumor cells infiltrating the muscularis propria) compartments of sporadic CRC, the deep compartments showing MMR protein down-regulation and increased MSI[41,44]. At least one-third of unstable tumors in deep compartments can be expected to be stable in superficial compartments. These differences can eventually result in the classification of a given tumor as MMS or MSI depending on the sample origin (superficial or deep).

CLINICAL TESTING FOR MMR DEFECTS

MSI results from the dysfunction of MMR proteins, which can be detected at genetic or protein levels. It is recommended that a CRC should be tested for MSI prior to gene testing, since this test is inexpensive and will help predict whether or not an individual has a germline MMR gene mutation[45,46]. Since up to 5% of HNPCC tumors do not have MSI, negative MSI tests cannot completely rule out HNPCC. Conversely, a positive MSI test is not diagnostic of HNPCC because 15%-30% of unselected CRC have MSI (due to *MLH1* promoter methylation), whereas only 1%-6% of all CRC are associated with detectable HNPCC mutations. If the tumor is MSI-positive, further analyses for MMR defects are recommended.

Genetic testing for MMR defects

MMR defects are due to either inactivating point mutations spread throughout the genes, therefore needing full-length sequencing, or promoter hypermethylation (especially *MLH1* in sporadic CRC). HNPCC is an autosomal dominant disorder caused by germline MMR gene mutations, in particular in *MLH1*, *MSH2*, *MSH6*, and *PMS2*. No strong genotype-phenotype correlations have been observed to date, but *MSH2* mutations do appear to be associated with more extracolonic manifestations than *MLH1* mutations. *MSH6* mutations are more common in endometrial tumors and *PMS2* mutations are especially

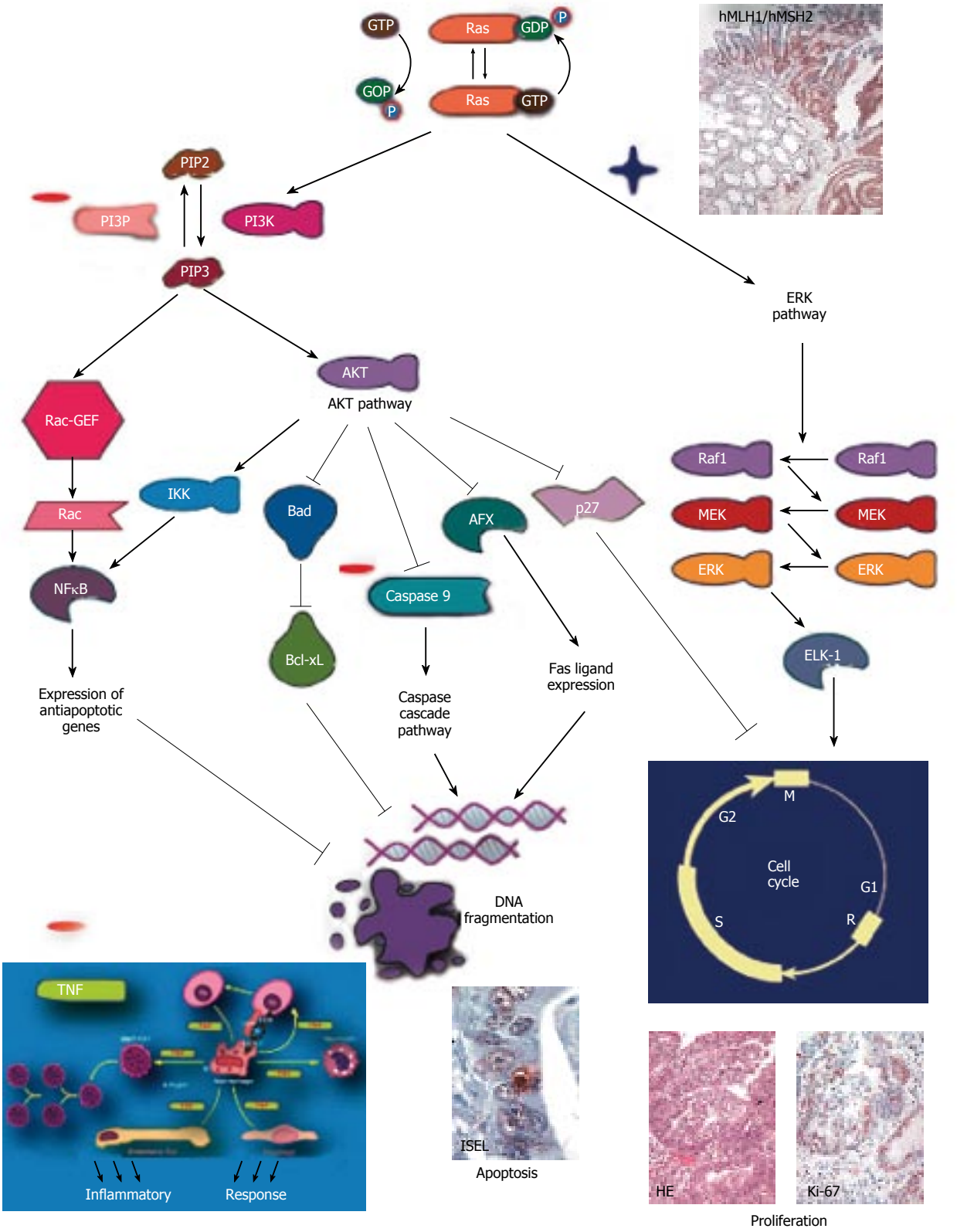


Figure 2 Molecular pathways contributing to the phenotype of microsatellite stable (MSS) CRC. MMR protein expression results in enhanced RAS signaling through ERK pathway (increased proliferation) and down-regulation of PI3P phosphatase, caspase 9 (apoptosis blockade), as well as TNF (decreased inflammation).

associated with Turcot's syndrome[47]. The original HNPCC diagnostic criteria were established by the International Collaborative Group on Hereditary Nonpolyposis Colorectal Cancer (ICG-HNPCC) and are known as the Amsterdam criteria[30], but only 50%-70% of HNPCC families meeting these criteria have been found to have germline *MSH2* or *MLH1* mutations[48]. The Amsterdam criteria were revised by the ICG-HNPCC in 1999 to include extracolonic cancers. The least stringent criteria are the Bethesda guidelines (more sensitive but less specific

than either the Amsterdam I or Amsterdam Ⅱ criteria in identifying HNPCC families with pathogenic mutations), which aim to determine which patients should have MSI testing[48]. These criteria propose MSI testing for:

Individuals with cancer in families that meet the Amsterdam criteria.

Individuals with two hereditary nonpolyposis colon cancer syndrome (HNPCC)-related cancers, including synchronous and metachronous colorectal cancers or associated extracolonic cancers (endometrial, ovarian, gastric, hepatobiliary, or small bowel cancer or transitional cell carcinoma of the renal pelvis or ureter).

Individuals with colorectal cancer and a first-degree relative with colorectal cancer and/or HNPCC-related extracolonic cancer and/or a colorectal adenoma: one of the cancers diagnosed by age 45, and the adenoma diagnosed by age 40.

Individuals with colorectal cancer or endometrial cancer diagnosed by age 45.

Individuals with right-sided colorectal cancer with an undifferentiated pattern (solid/cribriform) on histopathology diagnosed by age 45.

Individuals with signet-ring-cell-type colorectal cancer diagnosed by age 45.

Individuals with adenomas diagnosed by age 40.

The American Gastroenterological Association recommends genetic testing for HNPCC for individuals from families meeting Amsterdam criteria, as well as for individuals with two HNPCC-related cancers (for instance, colorectal and endometrial cancer) and individuals with colorectal cancer who have a first degree relative with an HNPCC-related cancer (or colorectal adenoma) where at least one was diagnosed before age 50[45,46,49]. Ideally, testing should first be offered to a family member with colorectal or endometrial cancer[26,45,48,49]. In some individuals, genetic analysis may be offered after prescreening for MSI in an HNPCC-related tumor specimen. Such prescreening should be offered where an HNPCC-related cancer is present in two individuals related by first-degree regardless of age of onset, or individuals with early-onset CRC regardless of family history. Genetic testing is indicated if MSI is present.

The majority (90%) of mutation-positive HNPCC cases are caused by mutations in *MLH1* or *MSH2*[1,9]. For this reason, the mutation analysis is generally performed for these two genes, *MSH6* being included in the analysis more recently. Although several methods can be used to detect these mutations, direct exon-by-exon gene sequencing is considered the gold standard. The sequencing should analyze each of the protein-coding regions of the *MLH1* and *MSH2* genes in their entirety, with all positive results being repeated for confirmation. Once a specific mutation that has been found in a relative by previous genetic testing, a test examining only the specific portion of the gene containing the known familial mutation can be offered to all family members.

There are some benefits and limitations of genetic testing for HNPCC. Relying solely on family history can underestimate the risk of developing cancer in mutation carriers and over-estimate risk in those who do not inherit the mutation. When an individual has a personal or family history that suggests the possibility of HNPCC, an important step is to determine whether the person is interested in genetic testing. Genetic testing for HNPCC can have important benefits for members of high-risk families who choose to be tested[50]. Those who are found to carry deleterious mutations can take steps to reduce their cancer risk, especially through earlier and more intensive surveillance or consideration of prophylactic surgery. Individuals with HNPCC-related CRC can undergo surgical management designed to address the increased risk of a second cancer.

In families in whom a deleterious mutation has been found, those who are mutation-negative can be spared the need for more intensive surveillance and intervention[50]. However, these individuals remain at risk for sporadic CRC and should be encouraged to adhere to age-appropriate general population screening guidelines.

Before consenting to genetic analysis, patients should also consider the limitations of testing. Currently, genetic testing cannot detect unusual mutations responsible for HNPCC, such as those occurring in MMR genes other than *MLH1* and *MSH2*. Therefore, a negative result in an individual who does not have a family member with a documented mutation must be interpreted cautiously. The test may also detect a variant of uncertain significance whose effect on cancer risk has not yet been established. In such situations, testing other family members for the specific variant to determine if it is associated with cancer may provide clarification of the significance[50].

Immunohistochemical testing for MMR defects

At the protein level, hMLH1/hMSH2 immunohistochemistry has a role in detecting MMR defects[51-53], with data suggesting that the effectiveness of immunohistochemical screening of the MMR proteins would be similar to that of the more complex strategy of microsatellite genotyping[54]. This technique can guide which gene to sequence and can help differentiating sporadic from hereditary mutations: hMSH2 loss is likely to be HNPCC, whereas hMLH1 loss could be HNPCC or sporadic CRC (*MLH1* promoter methylation). MMR proteins heterodimerize to function; the hMSH2 loss almost always accompanies hMSH6 loss and when hMLH1 is lost, generally so is hPMS2[55-57]. In addition, immunohistochemistry can miss functional loss; i.e. presence of the protein with antigen positivity in the absence of function. Several antibodies have been used for these analyses, but the most widely used are hMSH2 (clone FE11, Oncogene Research), hMLH1 (clones G168 728 and G168-15, BD Pharmingen), hMSH6 (clone 44, BD Transduction Laboratories), and hPMS2 (clone A16-4, BD Pharmingen, and polyclonal C terminus, Santa Cruz Biotechnology).

MMR immunohistochemical studies are based on a complete absence of at least one MMR protein[5,12,37,51-53,58-61]. But these studies do not consider the immunostaining topographic heterogeneity[41]. Since the MMR proteins function as heterodimers, it could be advocated to validate the immunohistochemical results of hMSH2/hMSH6 and

hMLH1/hPMS2. More studies are required to clarify the influence of this predictable tumor heterogeneity to select the appropriate sample for immunohistochemical and/or MSI analyses.

PROGNOSTIC AND THERAPEUTIC IMPLICATIONS OF MSI

The CRC microsatellite profile provides useful prognostic information[6,26,39], showing the patients with microsatellite-unstable neoplasms have a better overall survival rate and a modified response to conventional chemotherapy[62-67]. MSI also helps in predicting the treatment response of CRC[63,64,68], and could modify the chemotherapy protocols offered to the patients in the future[64], but these results should be applied with caution before this predictive tool is verified[64].

Molecular markers as predictive factors in treatment decisions have been developed in the last few years. The initial studies in sporadic CRC showed that the retention of heterozygosity at one or more 17p or 18q alleles in microsatellite-stable CRCs and mutation of the gene for the type Ⅱ receptor for TGF-β1 in CRCs with high levels of microsatellite instability correlated with a favorable outcome after adjuvant chemotherapy with fluorouracil-based regimens, especially for stage Ⅲ CRC[63,68]. However, most recent studies have revealed that fluorouracil-based adjuvant chemotherapy benefited patients with stage Ⅱ or stage Ⅲ CRC with MSS tumors or tumors exhibiting low-frequency MSI but not those with CRCs exhibiting high-frequency MSI[64]. The reasons for these responses must be related to the distinctive cell kinetics associated with MMR down-regulation (significantly increased apoptosis and decreased proliferation), which can certainly contribute to tumor cell resistance to conventional chemotherapy[40,41]. The topographic heterogeneity of sporadic CRC is a key element to explain the discrepant results reported[41]. This point has not been systematically addressed yet, but a homogeneous selection of the samples from the same topography must be considered in the molecular test design[25].

CONCLUSIONS

Many CRC show MSI, for which confirmatory analyses are warranted because of prognostic and therapeutic implications. Pathologists play a critical role in identifying microsatellite-unstable CRC, such as occur in young patients with synchronous or metachronous tumors and tumors with classic histologic features. In these cases, MSI testing and/or MMR immunohistochemistry are advisable, along with sequencing and genetic counseling if appropriate. Microsatellite analysis is an excellent functional and prognostic test, whereas MMR immunohistochemistry can guide gene sequencing but can result in false negatives (false negative in antigen positive neoplasms, especially cases with MLH1 promoter methylation). Direct exon-by-exon gene sequencing is considered the gold standard and should be used to analyze each of the protein-coding regions of the *MLH1* and *MSH2* genes in their entirety, although this technique will miss *MLH1* gene inactivation by promoter methylation. Finally, the selection of samples for molecular tests must be carefully designed considering predictable heterogeneity, such as topographic heterogeneity, to avoid misinterpretations.

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REVIEW

Probiotics and prebiotics in chronic inflammatory bowel diseases

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Abstract

The prokaryotic and eukaryotic cells of the colon exist in a highly complex, but harmonious relationship. Disturbances in this remarkable symbiosis can result in the development of inflammatory bowel diseases (IBD). Although the etiology of IBD is not entirely understood, it is known that the chronic inflammation of Crohn's disease, ulcerative colitis and chronic pouchitis are a result of an overly aggressive immune response to the commensal intestinal flora in genetically susceptible hosts. Recent studies have enhanced our ability to understand the interaction between the host and its intestinal microflora and the role the microflora plays in maintaining intestinal homeostasis. As we begin to understand the benefits conferred to the intestine by the microflora, the notion of modifying the composition of the bacterial load to improve human health has arisen. A significant body of research now exists investigating the role of probiotics and prebiotics in ameliorating chronic intestinal inflammation. This article will begin with an overview of the role of the commensal microflora in maintaining mucosal immune homeostasis, and how a dysregulated immune response to the intestinal microflora results in IBD. This will be followed by a summary of the use of probiotics and prebiotics in experimental and human IBD.

Key words: Colitis; Crohn's disease; Microflora; Immunity; Probiotics; Prebiotics

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INTRODUCTION

At birth, the gastrointestinal tract is a sterile environment. Initial exposure of the gut to microbes occurs during the birthing process from the maternal fecal and vaginal flora. Within a few months after birth, a relatively stable microbial population is established[1]. This abundant, diverse and dynamic intestinal microflora normally lives in a complex, symbiotic relationship with the eukaryotic cells of the mucosa. About 100 trillion bacterial cells benefit from the constant nutrient flow, stable temperature and niches for various metabolic requirements provided by the intestinal environment. Likewise, the host benefits from the ability of the intestinal microflora to synthesize vitamin K, exert trophic effects on intestinal epithelial cells, salvage energy from unabsorbed food by producing short-chain fatty acids, inhibit the growth of pathogens, sustain intestinal barrier integrity and maintain mucosal immune homeostasis. Studies from germ-free animals reveal that the absence of resident intestinal microflora results in significant alterations in intestinal structure and function, including slender villi, shallow crypts, low leukocyte count[2,3], a decrease in the number and density of Peyer's patches[4] and decreased stimulation of migrating motor complexes[5].

In their co-evolution with bacteria, vertebrates develop pattern-recognition receptors, which are activated by specific molecular patterns unique to bacteria, fungi and viruses that are absent in eukaryotes (lipopolysaccharides, peptidoglycan, ssRNA, muramyl dipeptide, flagellins, *etc*). These include the Toll-like receptors (TLRs) and nucleotide oligomerization domains (NODs). TLRs and NODs are critical for the initiation of innate immune defense responses. Activation of their signaling cascades usually results in the production of pro-inflammatory cytokines. TLR signaling also provides a link between innate and adaptive immunity, as TLR signaling results in the maturation of dendritic cells, which activate adaptive immune responses[6]. Although stimulation of these receptors in most parts of the immune system results in production of inflammatory cytokines, these ligands are not only tolerated by the gut mucosal immune system, but also essential for adaptation to intestinal bacteria and maintenance of homeostasis[7]. The tolerance to the intestinal microflora is not completely understood, but several aspects of commensal physiology have been defined which contribute to their inability to activate the immune system. Some commensal bacteria can modify

TLR ligands, resulting in a hypoactive immune response. For example, the endotoxic portion of LPS is pentacylated in many *Bacteroides* species, and has minimal toxicity[8]. An important feature of commensal bacteria is their inability to penetrate the intestinal epithelial barrier. If some of these organisms do penetrate, they are usually rapidly phagocytosed by the innate mucosal immune system. Indeed, in a healthy host, the systemic immune system appears to be ignorant of the intestinal microflora[9]. Maintaining tolerance to these intestinal bacteria is a remarkable accomplishment achieved by the mucosal immune system, and disturbances in this bacterial-epithelial homeostasis result in considerable deleterious effects on the host.

Role of the commensal flora in IBD

Although many studies have investigated the possibility of a single infectious agent causing Crohn's disease and ulcerative colitis, also called chronic inflammatory bowel disease (IBD), none has yet been discovered. The intestinal bacteria are now believed to be involved in the initiation and perpetuation of IBD. The prevailing theory explaining the development of IBD is that the adaptive immune system is hyper-responsive to the commensal intestinal microflora in genetically susceptible individuals[10]. This hypothesis is supported by several observations: most inflammation occurs in areas with the highest density of intestinal bacteria, broad spectrum antibiotics improve chronic intestinal inflammation, and surgical diversion of the fecal stream can prevent recurrence of Crohn's disease. Most importantly, despite differences in the pathogenesis of chronic intestinal inflammation, a consistent feature of many animal models of IBD (such as IL-10 knockout mice and HLA-B27 transgenic rats) is the failure to develop chronic intestinal inflammation when these animals are raised in germ-free conditions[11,12]. Dysbiosis is also observed in IBD patients. Adherent and intramucosal bacteria, particularly *Bacteroides spp*, *Escherichia coli* (*E. coli*) and *Enterobacterium spp* are more abundant in patients with Crohn's disease (CD) than in controls[13,14] (Figure 1). Bacterial overgrowth and dysbiosis are also associated with the development of chronic pouchitis, the inflammation of the ileal reservoir created after ileo-anal anastamosis following colectomy in ulcerative colitis (UC) patients[15]. In addition, several selected commensal bacterial species can induce and perpetuate colitis in genetically susceptible rodent models of chronic intestinal inflammation[16,17] (Figure 1).

Other immune dysfunctions in IBD include aberrant secretion of pro-inflammatory cytokines and chemokines by intestinal epithelial cells and mucosal immune cells[18-20], altered TLR4 signaling[21], defective antigen presenting cell function[22] and lack of T-cell apoptosis[23]. It is now clear that ignorance of the systemic immune system to commensal intestinal microflora is lost in IBD patients, as shown by enhanced and persistent cell-mediated and humoral immune responses to these bacteria[24].

At least 7 genetic loci conferring susceptibility to CD, ulcerative colitis (UC) or both, have been identified[25]. Interestingly, such susceptibility genes associated with CD involve polymorphisms of the NOD2 gene[26] (the pattern recognition receptor for muramyl dipeptide) which can result in hampered innate immune functions by impairment of TLR function[27], defective clearance of invasive bacteria by macrophages[28], and decreased production of defensins[29].

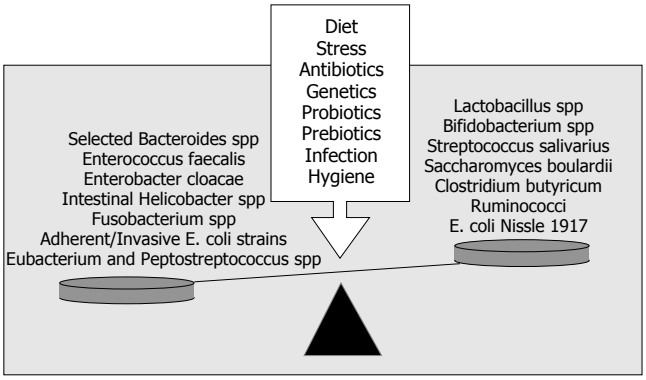

Figure 1 Microbial balance and dysbiosis. The pathogenic immune responses present in IBD are triggered by the presence of luminal bacteria. The balance of beneficial *vs* aggressive intestinal microbes is responsible for either mucosal homeostasis or chronic inflammation. A number of environmental and genetic factors influence the balance of beneficial *vs* aggressive microbes. Adapted from[63].

The recognition of the compelling association between intestinal microflora and the development of IBD has led to an abundance of studies investigating the therapeutic potential of altering luminal bacteria using probiotics and/or prebiotics.

Probiotics, prebiotics and synbiotics

Probiotics are defined as living organisms in food and dietary supplements which, upon ingestion, improve the health of the host beyond their inherent basic nutrition[30]. Probiotics are typically lactic acid bacteria selected from the gut flora, and are able to survive stomach acid and bile, maintain viability throughout extended periods of storage and are safe for human consumption. Other species have also shown some beneficial effects, such as *E. coli* Nissle 1917 and *Saccharomyces boulardii* (Figure 1). Probiotic bacteria have verifiable beneficial properties, including the ability to improve epithelial barrier function, modulate the mucosal immune system, and alter the intestinal flora.

Prebiotics are non-digestible dietary carbohydrates, e.g., lactosucrose, fructo- and galacto-oligosaccharides, inulin, psyllium, bran, germinated barley (Figure 2), which stimulate the growth and metabolism of endogenous enteric protective bacteria upon consumption. Beneficial effects of prebiotics are also associated with changes in colonic short-chain fatty acids (SCFA) due to fermentation by colonic bacteria[31]. Synbiotics are combinations of probiotics and prebiotics, and are also an emerging therapeutic modality. Restoration of normal microflora using probiotics, prebiotics or synbiotics has been investigated in numerous gastrointestinal and other disease states, including infectious diarrhea, *H pylori* infection, irritable bowel syndrome, colorectal cancer, lactase deficiency, pancreatitis, atopy, and IBD.

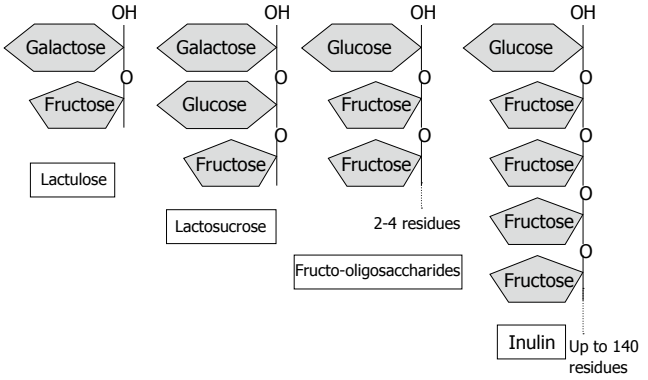

Figure 2 Basic structures of various prebiotic substances. Structurally, prebiotics are a mixture of polymers and oligomers comprising branching chains of fructose units.

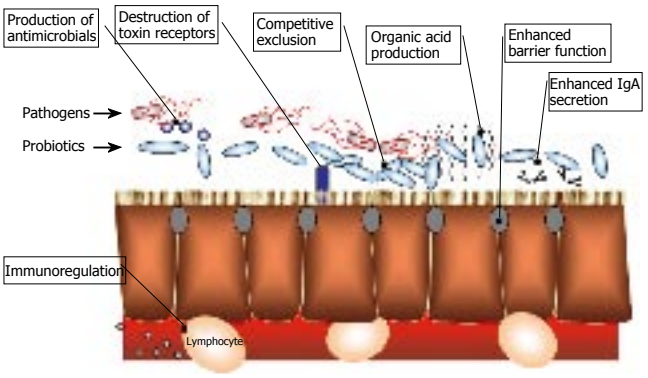

Figure 3 Mechanisms of probiotic activity.

Protective mechanisms of probiotics by ameliorating chronic intestinal inflammation

Probiotic bacteria have beneficial effects on the intestinal epithelia both directly and indirectly, including enhanced barrier function, modulation of the mucosal immune system, production of antimicrobials, and alteration of the intestinal microflora (Figure 3).

Alteration of the mucosal immune system. The presence of probiotics has been shown to result in several modifications in the mucosal immune response, including augmented antibody production[32,33], increased phagocyte[34] and natural killer cell activity[35-38], modulation of the nuclear factor kappa-B (NFκB) pathway[39-41], and induction of T cell apoptosis[42]. Generally, probiotics increase the production of intestinal anti-inflammatory cytokines (such as IL-10 and TGF-β), while reducing the production of pro-inflammatory cytokines (e.g., TNF-α, interferon-γ, IL-8)[43-46]. Several probiotic bacteria, including *B. breve*, *Streptococcus thermophilus*, *B. bifidum* and *Ruminococcus gnavus* have been shown to secrete metabolites that reduce LPS-induced TNF-α secretion[47]. *L. reuteri* reduces TNF-α and *Salmonella typhimurium* induceds IL-8 secretion *in vitro,* by inhibiting nuclear translocation of NFκB and preventing the degradation of IκB[48]. Administration of the probiotic cocktail VSL#3 (consisting of *L. acidophilus, L. bulgaricus, L. casei, L, plantarum, B. breve, B. infantis, B. longum, S. thermophilus*) to IL-10 deficient mice results in colitis reduction and a concomitant reduction in mucosal secretion of TNF-α and interferon-γ[49]. *E. coli* Nissle 1917 is able to down-regulate the expansion of newly recruited T-cells into the mucosa and limit chronic intestinal inflammation[50]. In SAMP1/Yit mice, *Lactobacillus casei* strain Shirota inhibits IL-6 production in LPS-stimulated large intestinal lamina propria mononuclear cells and down-regulates nuclear translocation of NFκB[51]. Patients with a recent ileo-anal pouch anastamosis who responded to probiotic therapy have reduced mRNA levels of IL-1β, IL-8 and IFN-γ, and fewer polymorphonuclear cells compared with patients who receive placebo[52]. Probiotic treatment has also been shown to reduce IFN-γ and IL1-α expression and decrease inducible-nitric oxide synthase and gelatinase activities in pouch biopsy samples from patients with pouchitis[53]. In mucosal explants of ileal specimens from patients with Crohn's disease, probiotics reduced TNF-α release and the number of CD4 cells[54]. In addition to live probiotics, components of probiotic bacteria can also exert effects on the mucosal immune system. For example, genomic DNA isolated from VSL#3 inhibits TNF-α-induced IL-8 secretion, mitogen-activated protein kinase activation and NFκB activation[41] in HT-29 cells.

Improved barrier function. Various probiotic bacteria can enhance intestinal epithelial barrier function. For example, oral administration of VSL#3 results in normalization of impaired colonic barrier function and restoration of intestinal epithelial integrity in IL-10 deficient mice and enhancement of epithelial resistance in T-84 cells[49]. Barrier function was enhanced not only by live bacteria, but also by a proteinaceous secreted product of VSL#3[49]. Several strains of lactobacilli are also capable of up-regulating intestinal MUC3 mRNA expression, thereby improving barrier function by increasing the mucus layer[55,56]. *Lactobacillus* GG (*L.* GG) improves barrier function by inhibiting apoptosis of intestinal epithelial cells[57]. *S. thermophilus* and *L. acidophilus* have been shown to enhance phosphorylation of actinin and occludin in the tight junction, thereby preventing the invasion of enteroinvasive *E. coli* into human intestinal epithelial cells[58].

Alteration of the intestinal flora. Probiotics suppress the growth and invasion of pathogens in several ways. They competitively exclude pathogenic bacteria by occupying the limited physical space in the mucus layer and on epithelial cells. They also engage pattern-recognition receptors and consume substrate otherwise available to other (pathogenic) microbes. In addition, probiotics render their microenvironment inauspicious for pathogens by secreting antimicrobial substances such as hydrogen peroxide, organic acids, and bacteriocins. For example, both *in vitro* and *in vivo* experiments demonstrate that *B. infantis* suppresses the growth of *Bacteroides vulgatus*[59]. VSL#3 has been shown to inhibit *Salmonella dublin* invasion into T-84 cells[49]. Patients with pouchitis treated with VSL#3 have been demonstrated to have increased bacterial diversity in the pouch, and decreased fungal diversity[60].

Probiotics may also alter the intestinal microflora by changing the fatty acid profile in the colon. VSL#3

Table 1 Clinical studies of probiotics in IBD

Author	Design	Group (Dose/d) (*n*) Probiotic	Comparator	Results
Induction of remission of ulcerative colitis				
Kato 2004 [87]	DB, R, C	Bifidobacterium fermented milk (100 mL) (10)	Placebo (10)	Reduced UCDAI ($P < 0.05$)
Rembacken 1999 [85]	DB, R, C	*E. coli* Nissle 1917 (1×10^{11} cfu) (57)	Mesalamine (59)	As effective as mesalamine at attaining remission
Bibiloni 2005 [88]	Open-label	VSL#3 (3.6×10^{9} cfu) (32)	None	77% remission or response rate
Ishikawa 2003 [86]	R, C	Lactobacillus and Bifidobacterium-fermented milk (100 mL) (11)	Placebo (10)	Reduced exacerbation of symptoms ($P < 0.01$)
Borody 2003 [90]	Case reports	Fecal enema (6)	None	100% remission
Maintenance of remission of ulcerative colitis				
Kruis 2004 [82]	DB, R, C	*E. coli* Nissle 1917 ($2.5\text{-}25 \times 10^{9}$ cfu) (162)	Mesalamine (165)	As effective as mesalamine at maintaining remission ($P = 0.003$)
Zocco 2006 [83]	Open-label	Lactobacillus GG (1.8×10^{10} cfu) (65)	Mesalamine (60) Mesalamine + LGG (62)	No difference in relapse rates at 12 mo. LGG more effective than mesalamine at prolonging relapse-free time ($P < 0.05$)
Shanahan 2006 [117]	DB, R, C	Lactobacillus salivarius or Bifidobacterium infantus (1×10^{9} cfu) (52/group)	Placebo (53)	No improvement of time to relapse
Venturi 1999 [84]	Open-label	VSL#3 (1×10^{12} cfu) (20)	None	75% maintained clinical and endoscopic remission
Induction of remission of Crohn's disease				
Schultz 2004 [97]	DB, R, C	Lactobacillus GG (2×10^{9} cfu) (5)	Placebo (6)	No difference in remission rates
McCarthy 2001 [118]	Open-label	Lactobacillus salivarius (1×10^{10} cfu) (25)	None	Reduced disease activity compared with baseline
Gupta 2000 [119]	Open-label	Lactobacillus GG (2×10^{10} cfu) (4)	None	Improvement in CDAI scores compared with baseline ($P < 0.05$)
Maintenance of remission of Crohn's disease				
Prantera 2002 [95]	DB, R, C	Lactobacillus GG (1.2×10^{10} cfu) (23)	Placebo (22)	No significant difference in remission
Campieri 2000 [120]	R, C	VSL#3 (3×10^{11} cfu) (20)	Mesalamine (20)	Equivalent to mesalamine in preventing recurrence
Marteau 2006 [94]	DB, R, C	Lactobacillus johnsonii LA1 (2×10^{9} cfu) (48)	Placebo (50)	No difference in endoscopic recurrence
Malchow 1997 [93]	DB, R, C	*E. coli* Nissle 1917 (5×10^{10} cfu) (16)	Placebo (12)	No difference in remission rates
Bousvaros 2005 [96]	DB, R, C	Lactobacillus GG (2×10^{10} cfu) (39)	Placebo (36)	No difference in time to relapse
Guslandi 2000 [91]	R, C 6 mo	Saccharomyces boulardii (1 g/d) + mesalamine (2g) (16)	Mesalamine (16)	Significant prolongation of remission ($P < 0.05$)
Induction of remission of pouchitis				
Kuisma 2003 [80]	DB, R, C	Lactobacillus GG (1×10^{10} cfu) (10)	Placebo (10)	No difference in PDAI
Laake 2004 [121]	Open-label	Lactobacillus acidophilus and Bifidobacterium lactis-fermented milk (500 mL) (51)	None	Improved PDAI, no difference in histology
Gionchetti 2000 [78]	DB, R, C	VSL#3 (6 g) (20)	Placebo (20)	Increased remission time ($P < 0.001$)
Mimura 2004 [122]	DB, R, C	VSL#3 (6 g) (20)	Placebo (16)	Increased remission time ($P < 0.0001$)
Gionchetti 2003 [79]	DB, R, C	VSL#3 (1×10^{11}) (20)	Placebo (20)	Increased remission time ($P < 0.05$)

DB: Double-blind; R: Randomized; C: Controlled; UCDAI: Ulcerative colitis disease activity index; CDAI: Crohn's disease activity index; LGG: Lactobacillus GG; PDAI: Pouchitis disease activity index.

probiotic strains are also capable of converting linoleic acid to conjugated linoleic acid, a fatty acid with anti-inflammatory and anti-carcinogenic properties[61].

Use of probiotics in inflammatory bowel disease treatment

Results from various animal studies and clinical trials using probiotics to treat intestinal inflammation have generated considerable excitement. Data are now emerging which suggest that probiotics are capable of preventing relapse of chronic intestinal inflammation. Some probiotics can even treat mild to moderately active IBD[62,63]. However, at present there is a relative lack of rigorously designed, randomized, placebo-controlled trials. Level 1 evidence is only available for the use of probiotics in post-operative chronic pouchitis while level 2 and 3 evidence supports the use of probiotics in treatment of CD and UC[62] (Table 1).

Experimental colitis

More than 20 animal models of IBD are available[64] and have been widely used to study the efficacy and mechanisms of probiotics in ameliorating inflammation in order to provide support for human clinical trials. In

IL-10 knockout mice, *L. plantarum* 299v[65], *L. reuteri*[46], *L. salivarius* subspecies *salivarius* 433118, *B. infantis* 35624[66], *L. salivarius* subspecies *salivarius* UCC118[67] and VSL#3[49] have all been shown to successfully attenuate intestinal inflammation. *L. GG* prevents recurrent colitis in HLA-B27 transgenic rats after antibiotic treatment, whereas *L. plantarum* has no effect[68]. Both VSL#3 and *L.* GG significantly ameliorate sulfhydryl-blocker iodoacetamide-induced colitis in rats, whereas they have no effect on dinitrobenzene sulfonic acid-induced colitis[69]. Improved inflammation in 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis has also been demonstrated after oral administration of *L. salivarius* ssp. *salivarius* CECT5713 and *L. plantarum* NCIMB8826[70,71]. Dextran sulphate sodium (DSS)-induced colitis in mice is ameliorated by soluble bacterial antigens extracted from *E. coli* (strain *Laves*) or by *Bifidobacterium* strains *breve*, *catenulatum*, and *longum*[72,73]. Daily administration of live but not heat-killed auto-aggregating *L. crispatus* reduces the severity of DSS-colitis in mice[74]. *L. reuteri* significantly reduces the colonic inflammation caused by both acetic acid and methotrexate in rats[75,76]. Interestingly, DNA from VSL#3 has been reported to reduce colonic inflammation, thus improving intestinal barrier function in IL-10 KO mice and DSS-induced colitis[41,77].

Chronic pouchitis

Probiotics can maintain antibiotic-induced remission in patients with chronic pouchitis after colectomy for refractory UC. Gionchetti *et al*[78] have completed placebo-controlled trials using the probiotic cocktail VSL#3 in patients with chronic relapsing pouchitis, showing that the remission rate after 1 year is 90% in the group treated with VSL3# versus 60% in the placebo group. Another study by the same group also showed that VSL#3 is also capable of preventing the development of chronic pouchitis during the first year after pouch surgery for UC[79]. Ten percent of patients who took VSL#3 developed pouchitis, compared with 40% of the placebo group. A double-blinded, prospective, randomized placebo-controlled trial was carried out in 20 patients treated with *L.*GG versus placebo for 3-mo[80]. In contrast to the study with the probiotic cocktail VSL#3, no significant differences were observed in chronic pouchitis disease activity in *L.*GG-treated patients despite an increased total fecal lactobacilli: anaerobe ratio, illustrating the inefficacy of *L.* GG for this condition.

Ulcerative colitis

Numerous studies have investigated the use of probiotics for maintenance of remission of UC in humans. A small study investigating the use of non-pathogenic *E. coli* Nissle 1917 versus low-dose mesalamine showed that it can maintain remission of quiescent UC, with a relapse rate of 16%-67% in those treated with *E.coli* versus 11%-73% in the mesalamine group[81]. In a double-blind, randomized trial involving 327 patients, Kruis *et al*[82] compared the effectiveness of an oral preparation of *E. coli* Nissle 1917 with mesalamine for maintaining remission of UC, and found that at the end of the 12-mo study, there is no significant difference between the two study groups, with relapses occurring in 36.4% of the *E. coli* Nissle 1917 group and 33.9% of the mesalamine group (significant equivalence, $P = 0.003$). Recently, Zocco *et al*[83] investigated the efficacy of *L.* GG in maintaining remission of UC and found that there is no difference in relapse rate between the 3 groups after 6 and 12 mo. However, *L.* GG is more effective than standard mesalamine treatment in prolonging relapse-free time ($P < 0.05$). An open-label study of VSL#3 showed that 15/20 UC patients remain in remission after 1 year[84].

Several studies have addressed treatment of established UC with probiotic therapy. A study using *E. coli* Nissle 1917 has demonstrated its equivalence to mesalamine for inducing remission of UC[85]. Ishikawa *et al*[86] evaluated *Bifidobacterium*-fermented milk in the treatment of UC, and symptoms of exacerbation were observed in 3 of 11 patients in the treated group versus 9 of 10 patients in the untreated group after 1 year ($P = 0.01$). However, no difference was observed in endoscopic disease activity. A placebo-controlled trial with bifidobacteria-fermented milk for 12 wk in UC patients with active disease showed that endoscopic disease activity index and histological score are significantly reduced in the treatment group compared with those in placebo group[87]. Uncontrolled administration of daily VSL#3 for 3 mo induces remission in 19 of 30 patients (63%), with a response rate of 87%[88]. As in all of these reported studies, the increased luminal probiotic bacteria return to baseline levels within one month after stopping probiotic treatment, indicating only transient colonization by these probiotic bacteria. In another small study administration of a combination of 3 bifidobacterium species for 2 mo was superior (20% remission) to placebo (93% remission) in maintaining remission of UC induced by sulfasalazine and glucocorticoids[89]. This effect correlates with decreased mucosal TNF-α, IL-1β and increased mucosal IL-10 levels[89]. An interesting study by Borody *et al*[90] showed that altering the gut microflora in UC patients achieved dramatic outcomes by administration of a freshly prepares enema from a healthy donor to six patients with relapsing refractory UC after broad spectrum antibiotics. This results in a remarkable reversal of all symptoms after 4 mo and sustained remission after 1-3 year of treatment.

Crohn's disease

Thus far, the use of probiotics for the prevention and treatment of CD is less substantiated than for the prevention and treatment of UC, although some studies certainly show promise. The effect of probiotics in maintaining remission of CD has been reported in an open-label study in patients receiving mesalamine alone versus mesalamine and *S. boulardii*[91]. At 6 mo, 37.5% of patients had a clinical relapse in the former group versus 6.3% of patients in the probiotic group. In an open-labeled study, McCarthy *et al*[92] reported that oral administration of *L. salivarius* UCC118 significantly reduces disease activity in patients with mild to moderate CD. In a randomized, placebo-controlled pilot study, patients with CD were treated with steroids and randomized to non-

Table 2 Clinical studies of prebiotics in IBD

Author	Design	Group (Dose/d) (*n*)		Results
		Prebiotic	Comparator	
Induction of remission of ulcerative colitis				
Kanauchi 2003 [112]	Open label	Conventional therapy + Germinated barley foodstuff (20-30 g) (21)	Conventional therapy	Improved UCDAI
Furrie 2005 [114]	DB, R, C	B. longum (2×10^{11}) + inulin/ oligofructose (6 g) (9)	Placebo (9)	Sigmoidoscopy scores reduced ($P = 0.06$) β-defensins, TNFα , IL-1α levels decreased ($P < 0.05$)
Maintenance of remission of ulcerative colitis				
Hanai 2004 [113]	Open label	Conventional therapy + Germinated barley foodstuff (20 g) (22)	Conventional therapy (37)	Improved UCDAI and endoscopic scores
Induction of remission of Crohn's disease				
Lindsay 2006 [115]	Open label	Fructo-oligosaccharides (15 g) (10)	None	Increase in IL-10 expressing intestinal dendritic cells

pathogenic *E. coli* Nissle 1917 as probiotic therapy or placebo[93]. After 1 year, there were fewer relapses in the probiotic group, but this was not statistically significant. Despite these promising studies, there are numerous reports on the inefficacy of some probiotics in CD. A randomized, placebo-controlled study of 98 patients showed that *L. johnsonii* LA1 is ineffective in preventing postoperative recurrence of CD[94]. A placebo-controlled trial with *L. GG* is also ineffective in preventing post-operative recurrence of CD in patients undergoing bowel resection[95]. Other studies have failed to detect benefits of *L.* GG in maintaining remission of CD in children[96] and adults[97,98]. Although *L.* GG has demonstrated its efficacy in treating rotaviral[99] and antibiotic-associated[100] diarrhea, results in IBD patients have been particularly underwhelming for this bacterial species, highlighting the species-specifity of colitis protection by probiotics.

PREBIOTICS IN INFLAMMATORY BOWEL DISEASE

Experimental colitis

Studies using prebiotics have been performed mostly in animal models. Lactulose and inulin have been shown to attenuate inflammation in IL-10 knockout mice and DSS-induced colitis respectively[46,101]. The combination of inulin and oligofructose (mixture 1:1) is also effective in preventing the development of colitis in HLA-B27 transgenic rats[102]. This beneficial effect is observed in conjunction with an increase of intestinal bifidobacteria and lactobacilli. Another study in HLA-B27 transgenic rats showed that the effects of the synbiotic "SIM", a combination of lactobacilli, bifidobacteria and the prebiotic inulin, are attributed to the inulin rather than the probiotics. The ingested probiotic bacteria are not detectable in the cecal content, yet the microflora profile of their cecal contents is altered[103]. In that study, inulin was also shown to specifically stimulate the growth of *Bifidobacterium animalis*. DSS-induced colitis rats fed with goat's milk oligosaccharides maintain their body weight, have reduced colonic myeloperoxidase activity and clinical symptoms and increased MUC-3 expression compared with control rats[104]. Goat's milk oligosaccharides also causes decreased anorexia, weight loss, bowel wall thickening and necrotic lesions in TNBS-induced colitis in rats, compared with untreated controls[105]. In another study of TNBS-induced colitis in rats, a 2-wk feeding of lactulose prior to the induction of colitis reduces myeloperoxidase activity, colonic TNFα and leukotriene B production, in conjunction with an increase of lactobacilli and bifidobacteria species in feces[106]. Lactulose has also demonstrated a dose-dependent beneficial effect on DSS-induced colitis in rats, including improvements of colonic ulceration areas, body weight changes, diarrhea, bloody stools and a reduction of myeloperoxidase activity and microscopic colitis[107].

However, not all studies using prebiotics have resulted in positive outcomes. Moreau *et al*[108] found that fructo-oligosaccharides are ineffective in improving DSS-induced colitis in rats. Holma *et al*[109] have reported a similar inefficacy of galacto-oligosaccharides in TNBS-colitis rats.

Several studies have investigated the use of an insoluble mixture of glutamine-rich protein and hemicellulose-rich dietary fiber termed germinated barley foodstuff (GBF). Fukuda *et al*[110] found that feeding GBF to rats with DSS-induced colitis resultes in significantly reduced colonic inflammation scores, and increased butyrate concentrations in cecal contents. Kanauchi *et al*[111] have observed similar results in DSS-colitis rats, and further determined that the dietary fiber component rather than the protein component of the GBF is responsible for the beneficial effects of GBF.

Ulcerative colitis

Table 2 lists the clinical trials using prebiotics to treat IBD. Although there is a paucity of human studies using prebiotics, the few emerging studies showed that there is potential for this treatment modality. A multi-centered open-label trial reported that oral administration of GBF to patients with mild to moderately active UC for 24 wk resultes in a significant decrease in clinical activity index, compared to controls[112]. An open-label study of 22 UC

patients in remission showed that a daily oral intake of 20 g GBF resultes in a significantly improved clinical activity index and endoscopic score at 3, 6 and 12 mo, and a reduced relapse rate, compared with controls[113]. A recent randomized, double-blinded controlled trial by Furrie *et al*[114] examined the use of synbiotics in 18 patients with active UC, using a combination therapy of *B. longum,* inulin and oligofructose, and found that sigmoidoscopy inflammation scores are reduced in the synbiotic-treated population when compared to placebo. Intestinal levels of TNF-α and IL-1β are also reduced. Additionally, rectal biopsies have demonstrated reduced inflammation and greater epithelial regeneration in the synbiotic-treatment group.

Crohn's disease

A small, uncontrolled study of 15 active CD patients reported that 21 d of fructo-oligosaccharide (15 g) intake results in a significant decrease of disease activity, an increase of intestinal bifidobacteria and modifications of Toll-like receptors and IL-10 expression in mucosal dendritic cells[115].

CONCLUSION

The link between intestinal microflora and IBD is now well established, and altering the composition of the microflora using probiotics and prebiotics holds promise as a therapeutic strategy for ameliorating chronic intestinal inflammation. Future developments in this field must include rigorous double-blind, placebo-controlled trials, using probiotics and prebiotics along with a further understanding of their protective mechanisms. Due to their excellent safety profile and lack of serious side effects, there are few contraindications to the consumption of prebiotics, probiotics and synbiotics by IBD patients. Further understanding of the interactions between microbes and gastrointestinal tract will help identify which strains of bacteria and/or which prebiotics may be effective in the treatment of different types of chronic inflammatory disease.

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REVIEW

Delayed gastric emptying is associated with pylorus-preserving but not classical Whipple pancreaticoduodenectomy: A review of the literature and critical reappraisal of the implicated pathomechanism

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Abstract

Pylorus-preserving pancreaticoduodenectomy (PPPD) is nowadays considered the treatment of choice for periampullary tumors, namely carcinoma of the head, neck, or uncinate process of the pancreas, the ampulla of Vater, distal common bile duct or carcinoma of the peri-Vaterian duodenum. Delayed gastric emptying (DGE) comprises one of the most troublesome complications of this procedure. A search of the literature using Pubmed/Medline was performed to identify clinical trials examining the incidence rate of DGE following standard Whipple pancreaticoduodenectomy (PD) *vs* PPPD. Additionally we performed a thorough in-depth analysis of the implicated pathomechanism underlying the occurrence of DGE after PPPD. In contrast to early studies, the majority of recently performed clinical trials demonstrated no significant association between the occurrence of DGE with either PD or PPPD. PD and PPPD procedures are equally effective operations regarding the postoperative occurrence of DGE. Further randomized trials are required to investigate the efficacy of a recently reported (but not yet tested in large-scale studies) modification, that is, PPPD with antecolic duodenojejunostomy.

Key words: Pylorus-preserving pancreaticoduodenectomy; Whipple pancreaticoduodenectomy; Delayed gastric emptying; Pancreatic surgery

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INTRODUCTION

The introduction of partial pancreaticoduodenectomy for the treatment of carcinoma of the ampulla of Vater dates back to almost a century ago and is credited to Kausch, a German surgeon from Berlin[1]. Then, in 1935 Whipple and associates redefined this procedure as a two-stage pancreaticoduodenectomy, where the pylorus and proximal duodenum are closed and preserved, while gastrointestinal continuity is re-established via a gastrojejunostomy[2]. Six years later, the first successful one-stage radical pancreaticoduodenectomy in which the distal stomach, pylorus and duodenum are removed, was reported independently by Whipple[3] and Trimble and coworkers[4]. Whipple is credited with popularizing the procedure, which now bears his name. Whipple pancreaticoduodenectomy (PD) has become the standard procedure of choice for many decades for the treatment of benign disorders requiring pancreaticoduodenectomy (such as chronic pancreatitis)[5], as well as for the treatment of periampullary tumors (carcinoma of the head, neck, or uncinate process of the pancreas, ampulla of the Vater, distal common bile duct, or peri-Vaterian duodenum)[6,7]. In 1978, Traverso and Longmire[8] reported a technique by which the whole stomach and 2.5 cm of the duodenum are preserved, restoring the gastrointestinal continuity by duodenojejunostomy. By application of pylorus-preserving pancreaticoduodenectomy (PPPD), the postgastrectomy syndrome (postprandial dumping, diarrhea, dyspepsia, nausea and vomiting) following Whipple resection is reduced and better functional results are achieved[9]. Although this technique has been initially reported by Watson[10] more than three decades before, the study by Traverso and Longmire[8] did not receive enough attention and has not been widely applied. In recent years, PPPD has been used increasingly by many surgeons, and is

considered the treatment of choice in many pancreatic surgery reference centers worldwide, despite the opinion that PPPD does not allow adequate resection of pancreatic or periampullary tumors[11,12].

A shorter operating time and a reduced intraoperative blood loss as a result of omission of gastric resection requiring the transfusion of fewer units of blood, as well as avoidance of PD-related dumping syndrome, better postoperative weight gain and a better quality of life, are considered advantages of PPPD over PD[8,13-20]. However, PPPD has been linked with a major drawback, that is, delayed gastric emptying (DGE), which is responsible for prolonged hospital stay and increased associated morbidity[11,21-23]. DGE has been reported in early studies to occur in up to 70% of patients undergoing PPPD proce dure[11,14,15,22-28]. Although the incidence of DGE appears to be declining in later published reports [5,29-31], DGE remains a leading cause of PPPD postoperative complications. The concern regarding an increased incidence of DGE following PPPD has prevented the adoption of this technique by some major American pancreatic centers[32].

Due to the fact that no uniform definition for DGE following pancreatic surgery exists, numerous controversial opinions have been reported regarding the efficacy of these techniques as causative factors for postoperative DGE occurrence. In an attempt to demonstrate which procedure, PD or PPPD, is preferable with regards to post-operative DGE occurrence, we performed a review of randomized, controlled trials in the English literature investigating the incidence of DGE following PPPD compared with PD. We further analyzed the various implicated pathomechanisms leading to the occurrence of DGE.

DEFINITION OF DGE

The occurrence of DGE following PPPD is initially reported by Warshaw and Torchiana[22]. In their study that included 8 patients undergoing PPPD, only 1 tolerated solid food within 10 postoperative days. Early studies have used a wide variety of definitions for DGE following pancreatic surgery. Some researchers have defined DGE as the inability to tolerate a regular or normal diet by the tenth[14,22,27] or fourteenth[26] postoperative day, or the start of a liquid diet after $\geqslant$ 7 d [25]. Others have described DGE as gastric stasis requiring gastric suction for 7 d[11,15] or $\geqslant$ 10 d[24,28].

In the recent years, although various definitions for DGE exist, 3 seem to be most widely acceptable. (1) According to the first definition[24], DGE occurs when the nasogastric tube is left in place for $\geqslant$ 10 d plus one of the following: emesis after removal of the nasogastric tube, reinsertion of a nasogastric tube, postoperative use of prokinetic agents after the 10th postoperative day, or failure to progress with diet. (2) According to the second definition[33], DGE occurs when nasogastric intubation is required $\geqslant$ 10 d following the operation, or is reinserted due to vomiting. (3) According to the third definition[34], DGE occurs when nasogastric intubation is required $\geqslant$ 10 d following the procedure or when a solid diet cannot be tolerated on or before the 14th postoperative day.

Independent of the correct definition, DGE not only leads to repeated episodes of nausea and vomiting, but also has an impact on postoperative weight gain, duration of hospitalization[26] and related morbidity, while it may also lead to fatal aspiration and pneumonia[35]. It is therefore a dangerous and potentially life-threatening complication.

WHAT IS THE PATHOMECHANISM UNDERLYING THE OCCURRENCE OF DGE?

A number of theories have been postulated to explain the occurrence of DGE after PPPD. Physiological gastric emptying and motility of the digestive system are complex processes that are controlled and regulated by complicated physiological mechanisms. Tonic contractions of the proximal stomach are important for the transfer of liquid food from the stomach to the duodenum[36,37], while peristaltic contractions of the distal stomach are of primary importance for reducing the size of the solid food particles and for the transfer of solid food to the duodenum[38]. Furthermore, certain properties of ingested food, such as volume, osmolality, pH and nutrient content, may down-regulate the motility of the digestive system, either via vagal and splachnic sensory pathways which mediate inhibition of gastric motility induced by duodenal distension[39], or *via* cholecystokin- and secretin-mediated pathways[40].

Cholecystokinin (CKK) has been shown in animals and humans to inhibit gastric emptying (especially the liquid-phase emptying of the stomach)[41] *via* a vagal capsaicin-sensitive afferent pathway and by stimulating phasic and tonic pyloric motility[42-45]. Muller and associates[46] reported that CCK levels decrease from 1.1 $\pm$ 0.2 pmol/L preoperatively to 0.8 $\pm$ 0.2 pmol/L 10 d postoperatively, and to 0.5 $\pm$ 0.1 pmol/L following PPPD, though no statistical significance could be demonstrated. The decrease in CCK levels is attributed to the resection of the duodenum, because high concentrations of CCK are found in the duodenal mucosa.

An alternative explanation is that the reduction of CCK levels is an adaptive response to DGE. Large amounts of CCK can also be released from the jejunum, as proven by the fact that bypassing of the duodenum in patients with Billroth Ⅱ gastrectomy does not decrease CCK secretion after ingestion of fats[47]. A finding that supports this theory is that blockage of CCK receptors with antagonists accelerates gastric emptying[48].

The role of plasma secretin levels in the development of DGE following PPPD remains controversial and has not yet been fully elucidated[46,49-50]. Another mechanism that has been demonstrated to influence food transit is CCK-mediated pancreatic polypeptide (PP) release, which is mainly controlled by vagal cholinergic mechanisms[51-53]. Studies in dogs and humans have shown that the duodenum and vagal innervation are necessary for normal postprandial release of PP from pancreas[54,55]. PPPD has been shown to be significantly associated with reduction of PP levels compared with the preoperative findings[46]. This reduction seems to be due to resection of the pancreatic head, where the majority of PP-producing cells

are located[54].

Other researchers support the theory that DGE occurs as a direct result of the removal of the duodenum, which influences gastric secretion and emptying as well as pancreatic and biliary secretion, thus playing an important role in the regulation of pancreatic hormone release[56,57]. In addition, duodenectomy disrupts the coordination of gastric and intestinal migrating motor complexes[58], decreases the postprandial PP release[59], and abolishes the interdigestive cycles of plasma PP[54]. Other investigators believe that preservation of the duodenal pacemaker located 0.5-1 cm distally from the pylorus should be the mainstay of the procedure, in order to avoid disturbances in normal gastric peristalsis[60]. Gastric dysrhythmias probably exacerbated by some intra-abdominal complications such as an anastomotic leak or an abscess have also been thought to be the causative factor for DGE following PPPD[61]. In addition, problems caused by the surgical procedure itself, namely the injury to the nerve of Latarjet, or placement of suture material through the pyloric muscle resulting in ischemia of the gastroduodenal segments and gastroparesis, have similarly been implicated[15].

Multiple other causative agents have been implicated as etiological factors for DGE after PPPD, namely intra-abdominal complications, such as a leakage or an abscess[16,26,61,62], postoperative pancreatitis[63], pancreatic fibrosis[64], preoperative cholangiitis[62], pylorospasm secondary to vagal injuries that requires the performance of pyloromyotomy[65], alternation of the endocrinologic millieau[15,19], early enteral nutrition commencing on the first postoperative day[66], and torsion or angulation of the reconstructed alimentary tract[23,67]. It has been advocated that preservation of the right gastric artery is essential for avoidance of DGE, because of its arterial supply to the pylorus and antrum[23], although there are other investigators who do not support this theory[64]. A more recent experimental study suggests that division of neurovascular supply to the pylorus and/or transection of the duodenum may lead to DGE following PPPD[68]. These investigators underlined that, besides the right gastric artery, additional preservation of the supraduodenal artery, as well as conservation of the pyloric branch of the vagus nerve, are crucial for avoidance of DGE following PPPD.

In an interesting study from two surgical institutes in the Netherlands[69], the choice of Billroth I (proximal end-to-end duodenojejunostomy) or Billroth Ⅱ (end-to-side pancreatojejunostomy at the end of the jejunal loop, followed by end-to-side hepaticojejunostomy and an end-to-side duodenojejunostomy) type of reconstruction has been shown to influence DGE after PPPD. Although significantly less procedure-related complications were noted following Billroth I compared to Billroth II type of reconstruction (18% *vs* 42% respectively, $P < 0.05$), DGE occurred in significantly more patients receiving Billroth I compared to Billroth Ⅱ type of reconstruction (76% *vs* 32% respectively, $P < 0.05$).

The type of reconstruction of pancreaticogastroin testinal continuity following pancreatoduodenectomy has also been implicated to play a significant role in the development of DGE. A randomized study comparing pancreaticogastrostomy [PG] (69 patients) *vs* end-to-side pancreaticojejunostomy [PJ] (82 patients)[70], showed that PG is superior regarding DGE rates (2 *vs* 10 patients, or 3% *vs* 12%, respectively, $P = 0.03$). By using the PG reconstructive technique (a single layer of nonabsorbable interrupted stitches on the posterior wall of the stomach)[71] instead of PJ (single layer pancreaticojejunal or duct to mucosa technique)[72], the authors found that significantly less complications occur (25% *vs* 68%, respectively, $P = 0.002$). More specifically, the lower rates of biliary fistulae (0% *vs* 8.5%, respectively, $P = 0.01$) and intra-abdominal fluid collections (10% *vs* 27%, respectively, $P = 0.01$) following PG compared with PJ, are the main culprits for the decreased rates of DGE.

Postoperative complications have been reported to correlate significantly with the occurrence of DGE in other trials as well. Horstmann and associates[73] showed that the incidence of DGE increases from 1% when no postoperative complications occur, to 28% and 43% respectively when moderate (wound infection, temporary cardiopulmonary complications, transient occurrence of amylase/lipase-rich drainage fluid without signs of sepsis) and severe (anastomotic leakage, bleeding, septic complications, reoperation) complications occur ($P < 0.0001$). The results of several other studies lend support to this theory[16,27,33,74-77].

An in-depth analysis of the physiology of the mechanism underlying the occurrence of DGE has also been reported, showing that the initiation of interdigestive phase Ⅲ is closely related to the elevation of plasma motilin concentration[78]. Motilin, a 22-aminoacid residue polypeptide, originates in motilin cells, which are scattered in the duodenal epithelium[78]. Erythromycin and related 14-member macrolide compounds act as motilin agonists by binding to motilin receptors, which are largely confined to the antrum of the stomach and the upper duodenum[79], thus initiating phase 3 activity of the interdigestive migratory motor complex (MMC)[80-81]. An early study[24] showed that patients administering high doses (200 mg) of erythromycin every 6 h from postoperative d 3 to 10 have a 53% reduction in the incidence of DGE compared with placebo.

Studies in unfed normal patients have shown that high doses of erythromycin (200-300 mg) induce strong, prolonged bursts of antral contraction, which are not propagated to the small intestine[82,83]. On the contrary, erythromycin administered in low doses (40 mg) induces premature phase 3, commencing in the stomach and migrating through the small intestine, which is similar to spontaneously occurring phase 3[82]. To test this hypothesis, Ohwada and coworkers[84] performed a prospective randomized, placebo-controlled trial investigating the effect of low-dose erythromycin *vs* placebo administration on DGE following PPPD and demonstrated that intravenous administration of erythromycin lactobionate (1 mg/kg) in 50 mL of 0.9% saline, given over 15 min through a central venous route every 8 h from postoperative d 1 to 14 results in reduction in the incidence of DGE following PPPD compared with placebo (14.3% *vs* 57.1% for erythromycin and placebo respectively, $P = 0.04$). Use of low-dose

erythromycin is significantly associated with induction of phase 3 of the MMC and initiation of phase 3-like contractions ($P < 0.0001$), earlier nasogastric tube removal ($P < 0.001$) and earlier progression to diet ($P < 0.003$). In contrast, the number of patients who had a nasogastric tube reinserted and emesis after nasogastric tube removal was similar in both groups. Still, erythromycin administration was associated with a 75% reduction in the incidence of DGE. In addition, a stepwise multiple regression analysis using a Cox proportional hazard model, showed that erythromycin and preservation of right gastric artery are significant covariates. Right gastric artery removal is a predictive factor for the effectiveness of erythromycin. The authors concluded that a low dose of erythromycin is not only more effective in reducing DGE after PPPD, but is also associated with a much lower rate of adverse effects compared with a high dose.

Octreotide, a long-lasting somatostatin analogue[85], administered preoperatively and continued postoperatively for 7 d at a dosage of 100 μg given subcutaneously 3 times a day has been reported to accelerate the rate of gastric emptying[86]. A randomized, placebo controlled trial in healthy volunteers[87], showed that administration of octreotide in the above-mentioned dosage can significantly accelerate gastric emptying compared to placebo ($P < 0.05$). It is hypothesized that this occurs as a result of the suppression of postprandial CCK release. A role in the prevention of DGE following pancreatic surgery is thus suggested. A randomized, placebo-controlled report[88], however, has questioned the role of octreotide in pancreaticoduocenectomy procedures. A similar study[89] showed that although octreotide use is associated with decreased rates of DGE compared with non-use, its use is significantly associated with the development of pancreatic fistulae. Based on their findings, the authors suggest avoidance of routine use of octreotide after pancreatico duodenectomies until the development of international guidelines.

IS DGE SIGNIFICANTLY ASSOCIATED WITH PPPD, BUT NOT WITH PD?

We searched the Medline/Pubmed database for clinical studies comparing the efficacy of PD *versus* PPPD with regards to DGE excluding publications not in the English language (Table 1). As a result, a total of 17 trials investigating the incidence of DGE after PPPD compared with PD are identified[6,12,20,25,34,63,66,73,77,90-97]. On the whole, 910 patients undergoing PD are compared with 1078 patients undergoing PPPD. Therefore, a total of 1988 patients have participated in these 17 studies.

Most early studies[12,25,63,90] showed that PD is superior to PPPD regarding incidence rates of DGE. However, only one study has demonstrated statistical significance in this outcome[25]. Another study showed that the difference in the occurrence rates of DGE after the two procedures is not significant because DGE when presents, resolves spontaneously within 6 wk. Later performed studies seem to support that the incidence rates of DGE following either PD or PPPD are comparable[34,63,66,73,77,91,93,95], although supporters of PD over PPPD regarding DGE rates also exist[94]. Some recent trials have even provided significantly lower rates of DGE following PPPD than following PD[92,96,97].

The reasons behind this diversity are multifactorial. The definition of DGE following pancreatic surgery varies from study to study. Improvement of surgical technique and increased surgical experience as well as advances in perioperative and critical care management, have resulted in decreased rates of DGE in recent years. The degree of lymph node dissection and pancreatic resection as well as the performance of anastomoses vary in different centers. Peri-operative administration of drugs that have been shown to decrease post-operational rates of DGE, like octreotide or erythromycin lactobionate, varies from study to study. The indication for performing PD varies significantly not only between different studies, but also within the same patient cohort. There is therefore a growing need for a multicentre, randomized clinical trial with specific guidelines for peri-operative administration of pharmaceutical agents, standard definition of the term DGE, and specific etiology-based performance of pancreatic surgery, to compare the efficiency of the two methods regarding DGE.

DISCUSSION

A recently reported modification in the classical PPPD procedure is the performance of duodenojejunostomy antecolically instead of retrocolically. Traverso and Kozuschek[98] reported a decade ago that antecolic duodenojejunostomy seems to be preferred by an increasing number of pancreatic surgery centers worldwide[61,69,99-101]. The theoretical background for this technique is that decreased blood circulation (especially venous drainage) of the jejunal limb following biliary-pancreato-enteric reconstructions can lead to decreased motility and profound edema of the jejunal limb itself, and eventually edema of the duodenojejunal anastomosis[27]. Compromised venous drainage of the jejunal limb, which is the peristalsis starting point of the newly constructed intestinal pathway, might lead to delayed recovery of jejunal peristalsis at the site of duodenojejunostomy, which will then cause DGE[62]. From a theoretical point of view, antecolic duodenojejunostomy avoids mechanical problems, because the descending jejunal loop is more mobile than after retrocolic reconstruction.

Kurosaki and Hatakeyama[99] evaluated the results of antecolic duodenojejunostomy in 55 consecutive patients undergoing PPPD as the selected mode of therapy for a wide variety of underlying diseases, and demonstrated that by use of the antecolic jejunal reconstruction method, DGE is markedly reduced based on the choice of the definition of DGE selected. According to the definition by Fabre *et al*[33], DGE occurs in only 5.5% patients. According to the definition by van Berge Henegouwen *et al*[34], DGE occurs in 29.1% patients, while according to the definition by Yeo *et al*[24], DGE occurs in 18.2% patients.

These researchers demonstrated that the development of a major complication is correlated significantly with reinsertion of nasogastric tube or emesis ($P = 0.010$), a later initiation of liquid diet ($P = 0.0381$) and a later

Table 1 Association between DGE and PD/PPPD

Study	Yr	Patients (*n*)	Results
Klinkenbijl *et al*[20]	1992	91 (44 PDs, 47 PPPDs)	No difference with regards to DGE was demonstrated between the two groups (i.e. days to liquid and normal diet)
Roder *et al*[12]	1992	110 (62 PDs, 48 PPPDs)	DGE was noted in 0 (0%) patients after PD and 9 (19%) patients after PPPD (*P* value not mentioned)
Patel *et al*[25]	1995	67 (52 PDs, 15 PPPDs)	DGE was noted in 41% of the PD group and 61% of the PPPD group ($P = 0.04$)
Mosca *et al*[90]	1997	218 (61 PDs, 157 PPPDs)	DGE was noted in 1 (4.7%) patient after PD and 14 (8.9%) patients after PPPD (*P* value not mentioned).
van Berge Henegouwen *et al*[34]	1997	200 (100 PDs, 100 PPPDs)	DGE was noted in 34 patients after PD and 37 patients after PPPD (P = NS)[1]
Lin and Lin[63]	1999	30 (15 PDs, 15 PPPDs)	DGE was noted in 1 patient after PD and 6 patients after PPPD ($P = 0.08$, two-sided Fisher's exact test, NS)
Di Carlo *et al*[91]	1999	113 (39 PDs, 74 PPPDs)	DGE was noted in 6 (15.3%) patients after PD and 9 (12.1%) patients after PPPD (P = NS)
Yeo *et al*[92]	1999	106 (58 PDs, 48 PPPDs)[2]	DGE was noted in 9 (16%) patients after PD and 2 (4%) patients after PPPD ($P = 0.03$)
Seiler *et al*[93]	2000	77 (40 PDs, 37 PPPDs)	DGE was noted in 18 (45%) patients after PD and 12 (32%) patients after PPPD ($P = 0.17$, NS)
Martignoni *et al*[66]	2000	62 (27 PDs, 35 PPPDs)	DGE was noted in 9 (33%) patients after PD and 13 (37%) patients after PPPD (P = NS)
Yamaguchi *et al*[94]	2001	50 (27 PDs, 23 PPPDs)	DGE was significantly associated with PPPD compared with PD (gastric tube removal, $P < 0.0001$, oral intake, $P = 0.0018$)
Yeo *et al*[6]	2002	294 (148 PDs, 146 PPPDs)	DGE was noted in 24 (16%) patients after PD and 9 (6%) patients after PPPD ($P = 0.006$)
Nguyen *et al*[95]	2003	105 (50 PDs, 55 PPPDs)[3]	DGE was noted in 6 of 50 (12%) patients after PD and 4 of 55 (7%) patients after PPPD ($P = 0.40$, NS)
Horstmann *et al*[73]	2004	132 (19 PDs, 113 PPPDs)[4]	DGE was noted in 4 of 19 (21%) patients after PD and 13 of 113 (12%) patients after PPPD ($P = 0.11$, NS)
Tran *et al*[77]	2004	170 (83 PDs, 87 PPPDs)[5]	DGE was noted in 18 patients after PD and 19 patients after PPPD ($P = 0.80$, NS)
Seiler *et al*[96]	2005	130 (66 PDs, 64 PPPDs)	DGE was noted in 30 (45%) patients after PD and 20 (31%) patients after PPPD ($P = 0.096$, NS)
Lin *et al*[97]	2005	33 (19 PDs, 14 PPPDs)[6]	DGE was noted in 6 (43%) patients after PD and 0 patients after PPPD ($P < 0.05$)

NS: Not significant. [1]Although nasogastric intubation was prolonged after PPPD *vs* PD (3 *vs* 6 d, $P < 0.0001$), this did not influence DGE rates; [2]Initially 114 patients were included in the study. Of these, 58 underwent PD while the remaining 56 were scheduled for PPPD. However, in 8 patients, the pylorus could not be preserved. They were therefore not included in the results; [3]In 7 of 55 (13%) patients in the PPPD group, the pylorus could not be preserved; [4]A total of 150 patients were included in the study but the 18 patients that underwent duodenum-preserving pancreatic head resection were not included here; [5]Two patients in the PPPD group were converted to the PD group during operation as the surgeon expected duodenal involvement; [6]Initially 36 patients were included in the study. Three patients with pancreatic head adencarcinoma initially assigned to the PPPD group, had to undergo PD eventually due to extensive duodenal involvement. These 3 patients were not calculated in either study groups.

progression to solid diet ($P = 0.0343$). Furthermore, a major complication is correlated significantly with DGE but only according to the definition of DGE by Yeo *et al*[24] ($P = 0.0006$), and not according to the definition of DGE by Fabre *et al*[33] ($P = 0.421$) or van Berge Henegouwen *et al*[34] ($P = 0.103$). A major complication is defined as a condition requiring invasive treatment or intensive care, or a pancreatic fistula proved by amylase-rich (> 1000 mg/dL) fluid from drains over 7 postoperative days or radiological examination. In their group, 10 patients developed a major complication (five patients required intensive care or invasive treatment, while another 5 developed a pancreatic fistula).

In addition, division of the left gastric vein (LGV) is correlated significantly with the occurrence of DGE (5.3% *vs* 37%, if the LGV is preserved or divided, respectively, $P = 0.0016$) according to the definition of DGE by van Berge Henegouwen *et al*[34] but not according to the definition of DGE by Yeo *et al*[24] (0% *vs* 5%, if the LGV is preserved or divided, respectively, $P = 0.067$). After summing up their results, the authors concluded that, by setting the stomach vertically in the left abdomen, antecolic duodenojejunostomy improves the occurrence of DGE after PPPD.

The decreased incidence of DGE following antemesenteric instead of retromesenteric jejunal reconstruction has been verified by other researchers as well. Park and associates[62] demonstrated that antemesenteric jejunal reconstruction is associated with a significantly lower incidence of DGE compared to retromesenteric reconstruction (6.5% *vs* 31.7%, respectively, $P < 0.05$) in terms of duration and amount of nasogastric drainage, as well as diet progression. Sugiyama and associates[100] also support the superiority of antemesenteric jejunal reconstruction with regards to DGE (8% *vs* 72% incidence of DGE for antemesenteric *vs* retromesenteric jejunal reconstruction respectively, $P < 0.001$). Horstmann and colleagues[73] demonstrated that performance of antecolic duodenojejunostomy-PPPD is associated with reduced (though not statistically significant) rates of DGE compared with standard Whipple (12% *vs* 21% for antecolic duodenojejunostomy-PPPD and

standard Whipple procedure respectively, $P = 0.11$).

So far, a major drawback of all reported studies is the lack of randomization. Their interpretation has therefore noticeable limitations. Recently however, two randomized controlled trials have verified the positive effect of the antecolic reconstruction method on DGE rates[102,103]. The first study[102] reported a significantly lower incidence of DGE after antecolic compared with retrocolic duodenojejunostomy (5% *vs* 50% respectively, $P = 0.0014$). However, due to the small number of patients included in this study ($n = 20$ patients/group), the authors support that larger-scale studies are needed to confirm the positive results of this new reconstruction method. The second study[103] demonstrated the same positive results (5.0% *vs* 24.0% for antecolic and retrocolic duodenojejunostomy, respectively, Odds Ratio: 0.167, 95% CI: 0.054-0.430, $P < 0.001$). Although the number of patients included in this trial was not as small ($n = 100$ patients/group)[103] as in the first study[102], a significant drawback is the difference in the time periods of sample collection (from January 1, 1996 until December 31, 2001 for the retromesenteric group, and from January 1, 2002 until December 31, 2003 for the antemesenteric group). Standardization of the operative technique, as well as continuous improvement in perioperative management, could account in part for the difference observed in DGE rates.

CONCLUSION

Pylorus-preserving pancreatic head resection and classical Whipple are equal operations regarding the postoperative development of delayed gastric emptying. Further randomized controlled trials are required to confirm the advantage of antecolic versus retrocolic duodenojejunostomy in PPPD.

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Induction of apoptosis on human hepatocarcinoma cell lines by an alkyl resorcinol isolated from *Lithraea molleoides*

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Abstract

AIM: To study the mechanism of cytotoxicity of a new active 5-alkyl resorcinol [1, 3-dihydroxy-5- (tridec-4', 7' -dienyl) benzene] isolated from *Lithraea molleoides* leaves on liver tumor cells.

METHODS: Human hepatocarcinoma cell lines (HepG2 and Hep3B) in culture were treated with inhibitory concentrations, 50% of the compound, for 24 h. The induction of apoptosis was detected in treated cells by analysis of DNA fragmentation, DNA content, and acridine orange and propidium iodide staining.

RESULTS: After 24 h of 5-alkyl resorcinol treatment, both cell lines showed: (1) the typical morphological alterations of apoptosis; (2) DNA fragmentation, detected by laddering and appearance of a subG0 population by flow cytometry; and (3) condensed and fragmented nuclei by acridine orange-propidium iodide staining.

CONCLUSION: Based on the results, this compound exerts its cytotoxic effect in both hepatocellular cell lines through apoptotic cell death. For Hep3B, cells with mutated p53 and Fas, apoptosis would proceed by p53- or Fas-independent pathways.

Key words: *Lithraea molleoides*; Cytotoxic activity; Alkyl resorcinol; Apoptosis; Hepatoma

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INTRODUCTION

Hepatocellular carcinoma is the 5th most common cancer in the world and the 4th most common cause of cancer-associated mortality[1]. Surgical resection and local treatment are frequently limited, as a result of metastasis, cirrhosis, and other pathological changes in the liver parenchyma. The development of chemotherapeutic or chemopreventive agents for hepatocellular carcinoma is important to reduce the mortality caused by this disease. Since cell homeostasis depends on the balance between proliferation and apoptosis, effective compounds inducing apoptosis appear to be a relevant strategy to suppress tumor growth[2]. Cytotoxic drugs cause cell death in sensitive cells, at least partly, by induction of apoptosis.

The Anacardiaceae family comprises many medicinal species from which a number of biologically active substances, such as various phenolic lipids (alkylresorcinols, alkylphenols, alkylcatechols), have been isolated. These compounds present antibacterial, fungicidal and cytotoxic properties[3,4]. In addition, cytotoxic activity on tumor cells (B-16, PC-13, L-5178Y, P-388 and Hep-2) and antitumor activity against S-180 tumors in mice have been reported[5,6]. *Lithraea molleoides* (Vell.) Engl, a member of the Anacardiaceae family, is a Southamerican tree that grows in Argentina, Brazil and Uruguay[7,8]. We have previously reported cytotoxic activity for the methanol extract of *L. molleoides* on HepG2 cells[9]. Further activity-guided fractionation of the dichloromethane extract has led to the isolation of a pure bioactive compound, a new cytotoxic 5-alkyl resorcinol (5-AR) derivative: 1,3-dihydroxy-5-(tridec-4',7'-dienyl) benzene[10].

The aim of the present study was to analyze the mechanism of cytotoxicity of this compound, by studying apoptosis induction on treated HepG2 and Hep3B hepatoma cell lines.

MATERIALS AND METHODS

Isolation and identification of the compound

The isolation and characterization of the *L. molleoides*

compound 1, 3-dyhydroxy-5- (tridec-4´, 7´-dienyl) benzene has been previously described[10].

Human tumoral cell lines

HepG2 and Hep3B cells, derived from human hepatoma, were obtained from the American Type Tissue Collection (ATCC, HB 8065 and HB 8064, respectively). They were cultured in minimal Eagle's medium (MEM) supplemented with 100 ml/L fetal bovine serum (FBS), 2 mmol/L glutamine, 1.5 g/L sodium bicarbonate, 1.0 mmol/L non-essential aminoacids, 1.0 mmol/L sodium pyruvate, and incubated at 37℃ in a humidified atmosphere containing 5% CO_2.

Cytotoxicity assay (MTT assay)

The cytotoxicity assay was carried out as previously described[9]. Briefly, 5×10^3 of HepG2 or Hep3B cells in growth medium were seeded in each well of a 96 well-microtiter plate, and incubated for 24 h at 37℃. Different concentrations of the 5-AR (in quadruplicates) were added to the exponentially growing cells. Cell controls in absence of the compound were included. After an incubation period of 24 h at 37℃, the MTT assay was performed following the manufacturer's instructions. The absorbance values at 546 nm and 650 nm were recorded in an ELISA plate reader (Meterech 960). The 50% inhibitory concentrations (IC50) for both cell lines were determined by linear regression from dose-response curves.

Analysis of DNA fragmentation

Approximately 1-2 $\times 10^6$ of control or treated cells were harvested and washed twice with PBS. DNA was extracted by the Wizard Genomic DNA Purification Kit (Promega), according to the manufacturer´s instructions. The DNA pellet was washed, air-dried, resuspended and electrophoresed on 1% agarose gel at 50 volts for 3 h. The gel was visualized under UV transilluminator and photographed.

Analysis of the DNA content by flow cytometry (hypoploid cells)

After a 24-h 5-AR treatment, control or treated cells were harvested and washed twice with 1 g/L bovine serum albumin (BSA) in PBS. They were fixed in 700 mL/L ethanol for 1 h at 4℃, washed twice with 1 g/L BSA in PBS and resuspended in the same buffer. After the addition of a DNA extraction buffer (192 mmol/L PO_4HNa_2; 4 mmol/L citric acid; pH 7.8), incubation at room temperature (RT) for 5 min and centrifugation, the cells were stained with propidium iodide (Sigma, 50 μg/mL) and treated with RNAse A (Sigma, 0.5 mg/mL) for 30 min at RT. Cells were analyzed on a flow cytometer (FACSCalibur, Becton Dickinson) containing an argon laser (488 nm). The red fluorescence of propidium iodide, proportional to the DNA content, was collected through a 620 ± 15 nm band pass filter. A minimum of 10000 cells per sample was collected and DNA hystograms were further analyzed by the WinMDI 2.8 program.

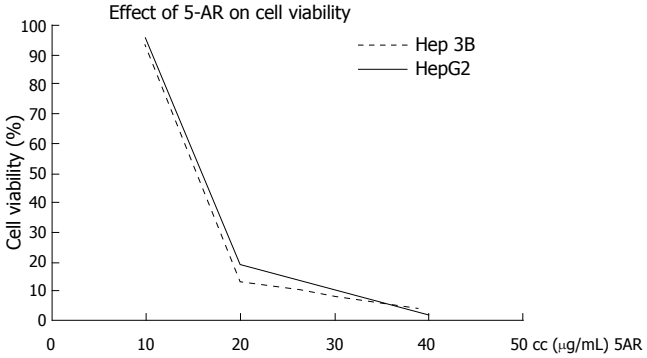

Figure 1 Treatment with 5-AR is highly cytotoxic for HepG2 and Hep3B cells. Cytotoxicity was studied testing cell viability by MTT assay in control or treated cells. The IC50 values for both cell lines were determined by linear regression from dose-response curves.

Acridine orange and propidium iodide staining

Approximately 1×10^6 of control or treated cells were resuspended in 5 mL of PBS containing 5 μg/mL of acridine orange (Sigma) and 5 μg/mL of propidium iodide (Sigma). The cell suspension was immediately dispensed onto slides, viewed under fluorescent microscopy (Nikon Eclipse 400) and photographed (Nikon Coolpix 4500).

RESULTS

Cytotoxitcity of 5-AR

The cytotoxicity of 5-AR on HepG2 and Hep3B human hepatoma cell lines was assessed by a cell viability assay, in the presence of different concentrations of the compound for 24 h. Under these experimental conditions, 5-AR exhibited a significant cytotoxic effect on both HepG2 and Hep3B cells. The IC50 values for HepG2 and Hep3B were interpolated from linear regression curves: 13.12 μg/mL (45.49 μmol/L) and 12.45 μg/mL (43.17 μmol/L), respectively (Figure 1). Both viability curves for 5-AR were practically overlapped, suggesting a parallel effect for cell death in both cell lines. This effect is independent of the p53 status of the cells, as cytotoxicity by 5-AR was similar for HepG2, which posses wild-type p53, and Hep3B, which are p53-deficient.

Based on the detected cytotoxic activity, the effect of the compound on cell apoptosis induction was examined in both cell lines by different methods.

Effect of 5-AR treatment on cellular morphology

Apoptosis, characterized by cell shrinkage, membrane blebbing, nuclear pyknosis, chromatin condensation, and genomic fragmentation, represents a universal and exquisitely efficient cellular suicide pathway. The cytoplasm condenses and cells shrink to finally form apoptotic bodies. HepG2 or Hep3B cells were treated for 24 h with the previously determined 24 h IC50 of the compound. After this period, the cells were observed under contrast phase microscopy (Nikon TMS) and photographed (Nikon FDX-35). The observation of cell morphology revealed that 5-AR-treated cells showed significant morphological

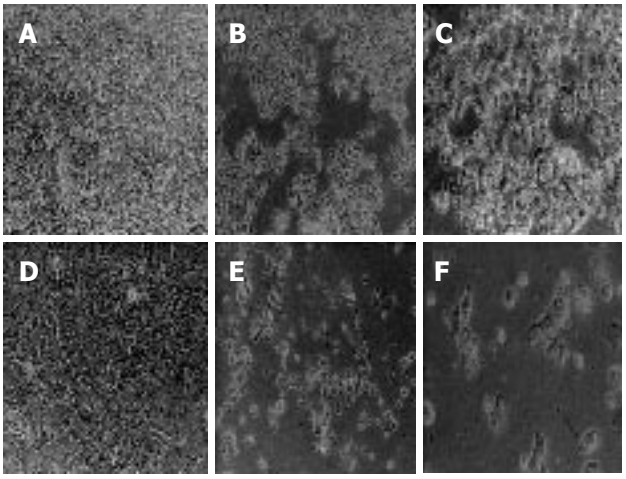

Figure 2 Treatment with 5-AR induces morphological changes typical of apoptosis in HepG2 and Hep3B cells. Control or treated cells were observed under contrast phase microscopy and photographed. **A:** Control untreated HepG2 cells (100 x magnification); **B:** HepG2 cells treated for 24 h with 5-AR IC50 (100 x magnification); **C:** HepG2 cells treated for 24 h with 5-AR IC50 (200 x magnification); **D:** control untreated Hep3B cells (100 x magnification); **E:** Hep3B cells treated for 24 h with 5-AR IC50 (100 x magnification); **F:** Hep3B cells treated for 24 h with 5-AR IC50 (200 x magnification). Results are from one experiment that is representative of three similar ones.

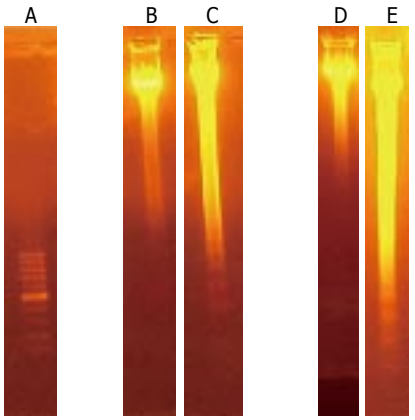

Figure 3 Treatment of HepG2 and Hep3B cells with 5-AR induces the DNA laddering typical of apoptosis. DNA was extracted from control or treated cells and electrophoresed on an agarose gel. Lane A: molecular marker; lane B: control untreated HepG2 cells; lane C: HepG2 cells treated for 24 h with 5-AR IC50; lane D: control untreated Hep3B cells; lane E: Hep3B cells treated for 24 h with 5-AR IC50. Results are from one experiment that is representative of three similar ones.

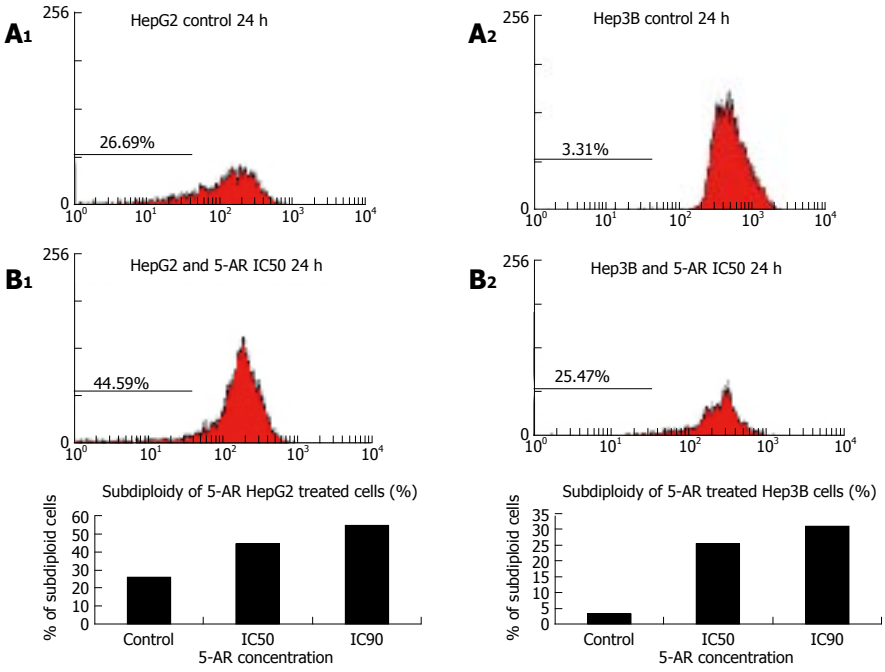

Figure 4 5-AR-treated HepG2 and Hep3B cells showing a subG0 population detected by flow cytometry. Treated or control cells were stained with propidium iodide and analyzed by flow cytometry. Left: (**A1**) control untreated HepG2 cells, (**B1**) HepG2 cells treated for 24 h with 5-AR IC50; Right: (**A2**) control untreated Hep3B cells, (**B2**) Hep3B cells treated for 24 h with 5-AR IC50. Results are from one experiment that is representative of three similar ones.

changes compatible with programmed cell death (Figure 2). The effects on cell morphology were dose-dependent (data not shown).

Effect of 5-AR treatment on DNA fragmentation

DNA fragmentation, the typical hallmark of apoptosis, was analyzed by DNA laddering on agarose gels and by the appearance of a subdyploid cell population with lower DNA content by flow cytometry.

The apoptotic process leads to fragmentation of cellular DNA in characteristic oligonucleosomal fragments, multiples of 200 base pairs. HepG2 and Hep3B cells, treated for 24 h with 5-AR, showed the typical DNA ladder on agarose gels (Figure 3). The intensity of this typical DNA ladder of multiples of 200 base pairs fragments was dose- and incubation time-dependent.

These results were further confirmed by flow cytometer hystograms obtained from treated cells, stained with propidium iodide. For HepG2 cells, the 5-AR treatment produced a significant increase in the percentage of the subG0 cell population (control cells: 26.09%; IC50: 44.59%). For Hep3B cells, the 5-AR treatment also produced a significant elevation of the subG0 cell population (control cells: 3.39%; IC50: 25.47%). The typical hypoploid population or subG0 peak, corresponding to cells with low DNA content, is shown in cytometry hystograms (Figure 4).

Induction of nuclear condensation by 5-AR treatment

Control or 5-AR-treated HepG2 and Hep3B cells were

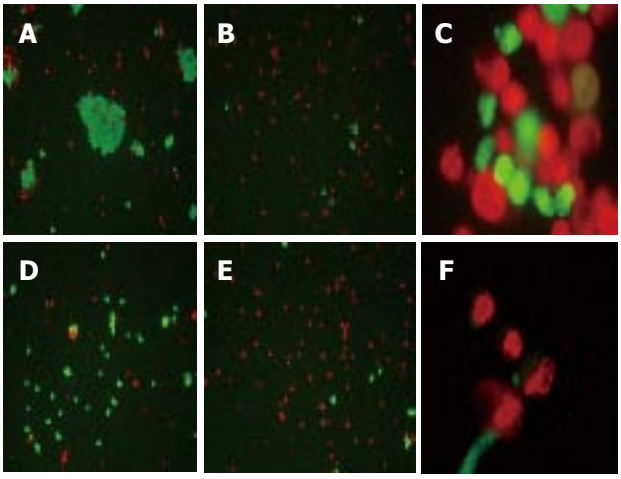

Figure 5 Treatment with 5-AR induces nuclear condensation and fragmentation in HepG2 and Hep3B cells. Cells were stained with acridine orange and propidium iodide, and observed under fluorescent microscopy. **A:** Control HepG2 cells (100 x magnification); **B:** treated HepG2 cells (100 x magnification); **C:** treated HepG2 cells (200 x magnification), showing in detail morphological changes of nuclear chromatin; **D:** control Hep3B cells (100 x magnification); **E:** treated Hep3B cells (100 x magnification); **F:** treated Hep3B cells (200 x magnification). Results are from one experiment that is representative of three similar ones.

stained with acridine orange and propidium iodide. A significant increase in the percentage of red cells and in condensed and fragmented nuclei were observed in treated cells, all these findings compatible with apoptotic cell death (Figure 5).

DISCUSSION

The search for novel anticancer drugs from natural sources has continued through the collaboration of scientists worldwide in looking for new bioactive compounds[12]. Experimental agents derived from natural products offer opportunities to evaluate not only totally new chemical classes of anticancer agents, but also novel and potentially relevant mechanisms of action[13].

With the aim of searching for new cytotoxic compounds from Argentine medicinal plants, a 5-AR was isolated from *L. molleoides*. The dose-dependent cytotoxic activity detected on many human tumoral cell lines suggested that it may contain some kind of antitumoral activity[10]. Due to the relevance of human hepatocarcinoma and the lack of available successful treatments, human hepatocarcinoma cells were selected to study the 5-AR cytotoxicity and to deepen into the mechanism of cell death induced by this compound.

Apoptotic pathways are involved in the cytotoxic mechanism of antitumoral drugs. Some anticancer drugs are known to upregulate Fas ligand, leading to its interaction with Fas and triggering the apoptotic pathway. On the other hand, p53 exerts its effects on cells as a transcription factor. An increase in p53 leads to the expression of pro-apoptotic proteins, which prompt cells to undergo apoptosis. It has been reported that p53-dependent apoptosis modulates the cytotoxicity of anticancer agents[14].

Considering the importance of Fas and p53 in hepatocyte cell death, two cell lines were selected based on their differential p53 and Fas phenotype. While HepG2 cells exhibit normal expression of both proteins, Hep3B cells present mutated p53 and Fas[15]. In this work, the treatment of HepG2 and Hep3B with IC50 doses of the 5-AR induced apoptosis in both cell lines, as evidenced by all the methodologies used in this work. Experiments with lower concentrations of the compounds also showed apoptosis induction in both cell lines (data not shown).

The fact that 5-AR can induce programmed cell death in Hep3B with non-functional p53[15] and Fas[16] evidences that the cytotoxic effect of 5-AR in this cell line is independent of their p53 or Fas phenotypic profile. As *p53* is the most common mutated gene in hepatocellular carcinomas, it is important to have cytotoxic compounds that exert their apoptotic activity in a p53-independent pathway to treat this kind of tumors.

The induction of apoptosis is known to be an efficient strategy for cancer therapy[17]. Recently, many plant extracts have demonstrated to posses the ability of triggering the apoptotic pathway[18,19]. The present study demonstrates that a pure compound isolated from *L. molleoides* is highly cytotoxic and presents apoptogenic activity on human hepatocarcinoma cell lines. The molecular mechanistic pathway involved in this process will be studied in our future experiments. Our results and the previously reported cytotoxic and antitumoral activities of other alkyl resorcinols justify further *in vitro* and *in vivo* studies to evaluate the potential use of this 5-AR as an antitumoral agent for hepatocellular carcinoma.

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VIRAL HEPATITIS

Distribution of hepatitis B virus genotypes: Phylogenetic analysis and virological characteristics of Genotype C circulating among HBV carriers in Kolkata, Eastern India

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Abstract

AIM: To evaluate the genotype distribution of hepatitis B virus (HBV) in Eastern India and to clarify the phylogenetic origin and virological characteristics of the recently identified genotype C in this region.

METHODS: Genotype determination, T1762/A1764 mutation in the basal core promoter (BCP) and A1896 mutation in the precore region of 230 subjects were determined by restriction fragment length polymorphism method (RFLP) and the result was confirmed by direct sequencing.

RESULTS: The predominant genotypes D (HBV/D) and A (HBV/A) were detected in 131/230 (57%) and 57/230 (25%) samples. In addition, genotype C (HBV/C) was detected in 42/230 (18%) isolates. Surface gene region was sequenced from 45 isolates (27 HBV/C, 9 HBV/A and 9 HBV/D). Phylogenetic analysis revealed that all of the HBV/C sequences clustered with South East Asian subgenotype (HBV/Cs). The sequence data showed remarkable similarity with a Thai strain (AF068756) (99.5% ± 0.4% nucleotide identities) in 90% of the genotype C strains analyzed. T1762/A1764 mutation in BCP region, associated with high ALT was significantly higher in HBeAg negative isolates than HBeAg positive isolates. Frequency of A1896 mutation leading to HBeAg negativity was low.

CONCLUSION: The present study reports the genotypic distribution and the characteristics of partial genome sequences of HBV/C isolates from Eastern India. Low genetic diversity and confinement of HBV/C in Eastern India possibly indicate a recent, limited, spread in this region. Genotype C with T1762/A1764 mutation has been reported to increase the risk for hepatocellular carcinoma; therefore genotype C carriers in Eastern India should be carefully monitored.

Key words: HBV genotypes; HBV/Cs, Eastern India; T1762/A1764 mutation

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INTRODUCTION

Hepatitis B virus (HBV) belongs to the *Hepadnaviridae* family of enveloped viruses with double-stranded DNA genome of nearly 3200 bp lengths. The HBV genome consists of four major overlapping open reading frames named surface (S), core (C), polymerase (P), and X.

HBV that infects humans has been classified into mainly eight genotypes, A-H based on the sequence divergence over the entire genome exceeding 8%[1-4] and S gene sequence analysis[5]. HBV genotypes have distinct geographical distributions and according to various studies seem to have different biological properties affecting, thus, the clinical outcome of HBV disease. Subgroups have been identified within different HBV genotypes, on the basis of > 4% (but < 8%) difference in the complete nucleotide sequence. In HBV genotype A, two subgroups have been defined; one is prevalent in Europe (Ae), and the other is prevalent in Africa and Asia (Aa)[6]. Similarly, genotype C has been classified into four subgroups with characteristic

geographical distributions. Subgroup C1 (Cs) is common in Southeast Asian countries like Thailand, Myanmar and Vietnam, C2 (Ce) in East Asian countries like Japan, Korea and China[7,8], C3 in Oceania comprising strains specifying *adrq-,* and C4 specifying *ayw3* is encountered in Aborigines from Australia[9,10].

Based on antigenic typing and further analysis of sub determinants HBV has been classified into 9 subtypes[11]. Subtypes correlate broadly with genotypes. Some subtypes can be found in more than one genotype, which confer additional heterogeneity within the genotypes.

It is now recognized that mutations in the basal core promoter (BCP) and precore region regulate hepatitis B e antigen (HBeAg) expression. It was reported in an *in vitro* study that the double mutations in BCP A1762T and T1764A (T1762/A1764A) down regulate precore mRNA and slightly increase the efficiency of pregenome mRNA and core mRNA[12]. Recently HBV genotypes have been partially clarified as influencing the clinical manifestation of chronic liver disease in hosts. A higher disease inducing capacity of HBV/C than HBV/B has been observed in Asia. Moreover, HBV/C with T1762/A1764 mutation has been reported to increase the risk for hepatocellular carcinoma[13].

India is a vast country with an ethnically diverse population. With more than 40 million carriers of HBV, this is the major etiology of chronic liver diseases in India. Analyses of genomic sequences of HBV isolates from India are limited. Most reports are from Western India and Northern India, where genotypes D and A are found[14,15]. Eastern India is a geographical area where genotypes D and A of mainland India and genotypes B and C of China and Southeast Asia converge. Recently genotype C has been reported from Eastern India[16-18]. However, data regarding genotype distribution as well as molecular and virological characteristics of genotype C in Eastern India remain undefined.

Therefore the present study was undertaken to investigate the distribution of HBV genotypes in Eastern India and the molecular and virological features of HBV/C circulating in Eastern India.

MATERIALS AND METHODS

Patients

A cross sectional study was performed on 230 HBV DNA positive serum samples from patients with HBV infection who were referred to Indian Council of Medical Research (ICMR) Virus Unit for HBV DNA detection. Among them, 200 samples came from the outpatient clinics of Kolkata hospitals during the period of March 2001 to February 2004. All patients were known to have been positive for surface antigen (HBsAg) for > 6 mo. In addition 30 HBsAg positive asymptomatic carriers found during a community based epidemiological point prevalence study carried out by Institute of Post Graduate Medical Education & Research (IPGMER) in the rural areas, about 150 km away from Kolkata, were also included in this study[19]. These samples were sent to our Unit for HBV DNA detection and for genotyping. Only pretreatment samples were included in the study. Informed consent was obtained from the patients, and the Institutional Ethical Committee approved of the study protocol.

Serological testing

HBsAg, HBeAg, antiHBe were tested by using commercially available enzyme linked immunosorbent assay kits (Organon Teknika, Boxtel, The Netherlands).

HBV DNA preparation and amplification

The sera were stored at -80℃ until analysis. Viral DNA was extracted from 200 μL serum by phenol/Chloroform extraction after incubation with Proteinase K[20]. HBV DNA was detected using in-house nested polymerase chain reactions (PCR) targeting the DNA sequences encoding the surface and the precore/core regions by the method described earlier[16, 17]. Instructions to prevent cross contamination were followed strictly[21] and the results were considered valid only when they were consistently obtained in duplicate.

Genotyping and Sequencing of HBV

HBV genotyping was done in 230 HBV DNA isolates by Restriction Fragment Length Polymorphism (RFLP) using the methods described earlier[5] and reconfirmed on the basis of phylogenetic analysis of the 440 nt fragment of S gene of 45 randomly chosen isolates. Of these 45 isolates, 27 were from genotype C, 9 from genotype A and 9 from genotype D. The nested PCR products were sequenced from both directions on an ABI Prism 377 (Applied Biosystems, Foster City, California, USA), using the PCR primers. BCP and precore core region were sequenced using primers as described earlier[17].

Subgenotypes of HBV/C were assigned as described previously, C1 (Cs), C2 (Ce)[7, 8] and C3, C4[10].

Phylogenetic analysis

For sequence alignment as well as phylogenetic analysis, we selected the GenBank sequences with the best and the high scoring matches with our sequences in a NCBI BLAST search. Sequences were edited, aligned and analyzed using Bioedit version 7.0.4.1[22]. Genetic distances were calculated using the Kimura two parameter algorithm and phylogenetic trees were constructed by the neighbor joining (NJ) method. To confirm the reliability of the pair wise comparison and phylogenetic tree analysis, bootstrap resampling and reconstruction were carried out 1000 times. Phylogenetic analysis was done using MEGA version 2.1[23].

RESULTS

The clinical and demographic characteristics of 230 HBV carriers are shown in Table 1. Among them 200 patients (age between 5-68 years, 77 HBeAg+ and 123 HBeAg-) were native resident of Kolkata and its neighborhood, while 30 (age between 2-50 years, 3 HBeAg+ and 27 HBeAg-) were from rural areas. All the patients were ethnic Bengali. Median serum HBV DNA load of the patients was 5.37 (range 5.15-9.15) log copies/mL.

Table 1 Clinical and demographic characteristics of 230 HBV carriers from Eastern India

	HBV carriers from		Total
	Kolkata (n = 200)	Rural areas (n = 30)	
Age (yr)	32.56 ± 12.98	26.03 ± 14.14	31.71 ± 13.29
Sex (M/F)	173/27	21/9	194/36
Genotype (A/C/D)	57/40/103	0/2/28	57/42/131
ALT (IU/L)	83.24 ± 75.15	37.63 ± 21.73	77.29 ± 72.15
HBeAg positive	77	3	80
HBeAg negative	123	27	150

Table 2 Characteristics of 42 HBV/C isolates of Eastern India

	HBeAg positive (n = 23)	HBeAg negative (n = 19)
Age (yr)	32.67 ± 13.04	32.47 ±13.01
Sex (M/F)	19/4	15/4
ALT (IU/L)	75.13 ± 39.50	85.84 ± 54.14
Subtype adrq+/adw2	23/0	18/1
T1762/A1764	12 (52%)	17 (89%)
A1896	2 (9%)	4 (21%)

Genotype distribution

Three HBV genotypes (A, C and D) could be detected among the 230 HBV DNA positive samples studied by RFLP method. Majority of the samples belonged to Genotype D, 131/230 (57.0%), followed by genotype A, 57/230 (24.8%) and 42/230 (18.2%) isolates were identified as genotype C, the majority of which (40 of 42) were from outpatient clinics of Kolkata. Out of the 30 samples from incidentally detected asymptomatic carriers from rural areas, only 2 (2/30, 6.7%) were of genotype C, while the rest were of genotype D (93.3%) (Table 1). The banding pattern of all the genotype C samples was similar to that of genotype C pattern found among Southeast Asian carriers by Lind *et al* 1997.

Characteristics of 42 HBV/C isolated from Eastern India

Among the 42 HBV/C strain, 23 (53%) were HBeAg positive and rest 19 (47%) were antiHBe positive. In order to clarify the clinical characteristics of HBV/C carriers in this region clinical and laboratory data between HBeAg positive and antiHBe positive patients were compared (Table 2). There was no significant difference between the mean age of HBeAg positive and antiHBe positive group. The mean ALT level was slightly high in antiHBe positive (85.84 ± 54.14 *vs* 75.13 ± 39.50) cases but the difference was not statistically significant. T1762/A1764 double mutation in the BCP region was more frequent in antiHBe positive than HBeAg positive group (17/19, 89% *vs* 12/23, 52%) with elevated ALT level, whereas the frequency of A1896 mutation that creates a stop codon in the precore region was low 6/42 (14%). To examine the correlation between age and HBeAg/antiHBe status, the age specific prevalence of the HBeAg/antiHBe status in 42 HBV/C subjects was analyzed (Figure 1). About 19/42 (45%) of subjects were in the age group of 16-30 years irrespective to HBeAg status. After that the infection rate gradually decreased.

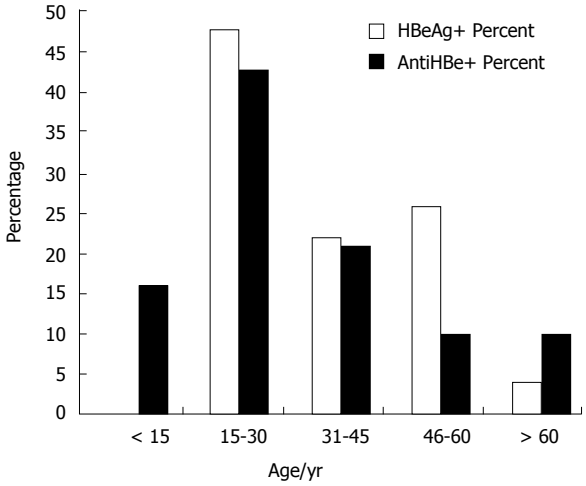

Figure 1 Age specific prevalence of HBeAg/antiHBe status in 42 HBV/C strain isolated from Eastern India.

Phylogenetic analysis

Phylogenetic analysis based on nucleotide (nt.) 256-696 of the Surface (S) gene region of HBV isolates was used to confirm the presence of these three genotypes from 45 isolates. The 45 S gene fragments were analyzed along with 39 reference sequences of different genotypes retrieved from GenBank in the phylogenetic tree presented in Figure 2. Genotypes A with adw2 subtype and D with ayw2 and ayw3 subtype from the present study clustered with the genotype A and D sequences previously reported from India. However, genotype C isolates with adrq+ and adw2 subtype from Eastern India clustered with HBV/Cs subgroup found in South East Asian countries rather than HBV/Ce subgroup found in the Far East like China, Japan, and Korea. Overall percent nucleotide identity (PNI) for HBV genotype C isolates from Eastern India with HBV/Cs varied from 98.4 to 100%. In addition to the above observation, 24 strains were most closely related to the Thai strain, AF068756 in the NCBI BLAST search. The PNI for these 24 sequences with AF068756 was found to be 99.5% ± 0.4%, across the 440 nt fragment in the S gene region. EI01343 and EI01329 were two HBV/C strain isolated from rural areas. Sequences have been submitted under GenBank accession numbers AY879184-AY879228.

Figure 3 represents the phylogenetic relatedness based on core sequences of the HBV/C strain from Eastern India. In this study, the core gene sequences were obtained from 19 HBV/C strain, 12 in this present study (accession no AY967430, AY967435, AY967442, AY967456-62, AY967465, AY967467) and 7 from previous study[17]. The phylogenetic tree was also constructed using the core gene sequences (nt. 1900 to 2350) of 39 reference sequences of different genotypes retrieved from GenBank along with 19 HBV/C strain from Eastern India. It was evident that all

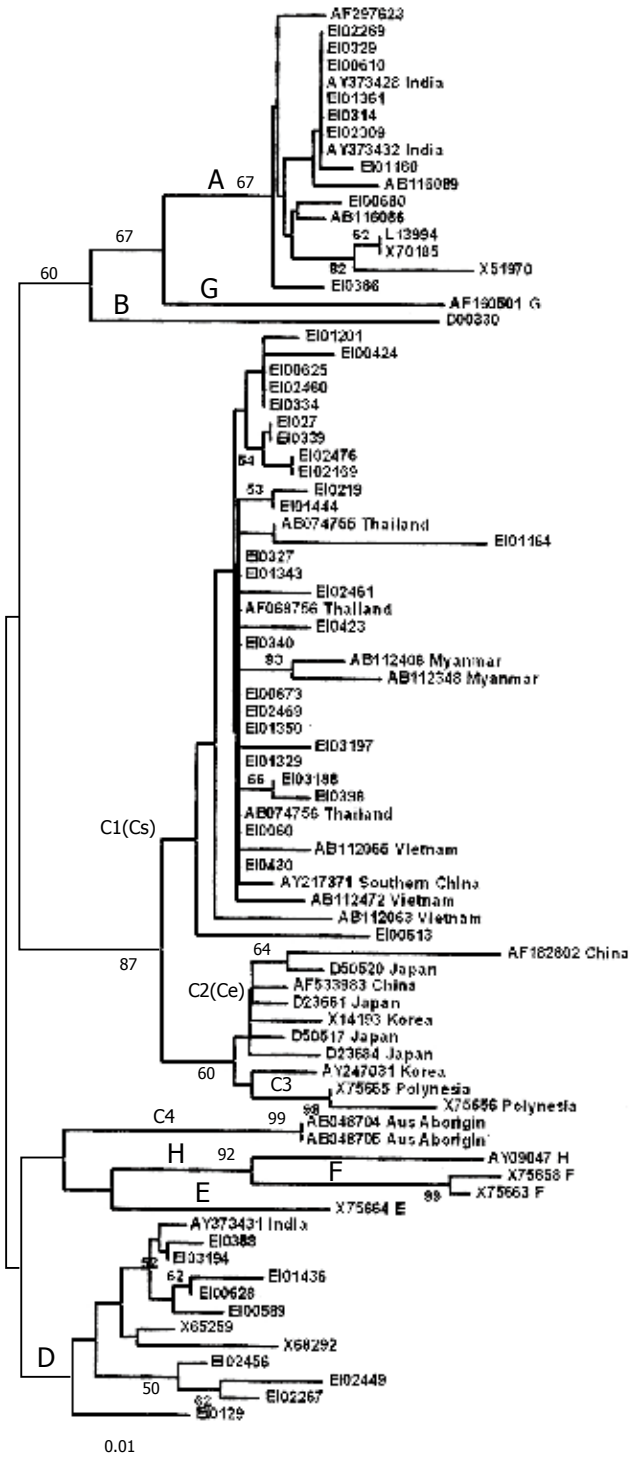

Figure 2 Neighbor joining phylogenetic of S gene region (codon 35-180) of HBV isolates from eastern India (denoted with EI) along with other genotypes derived from Genbank.

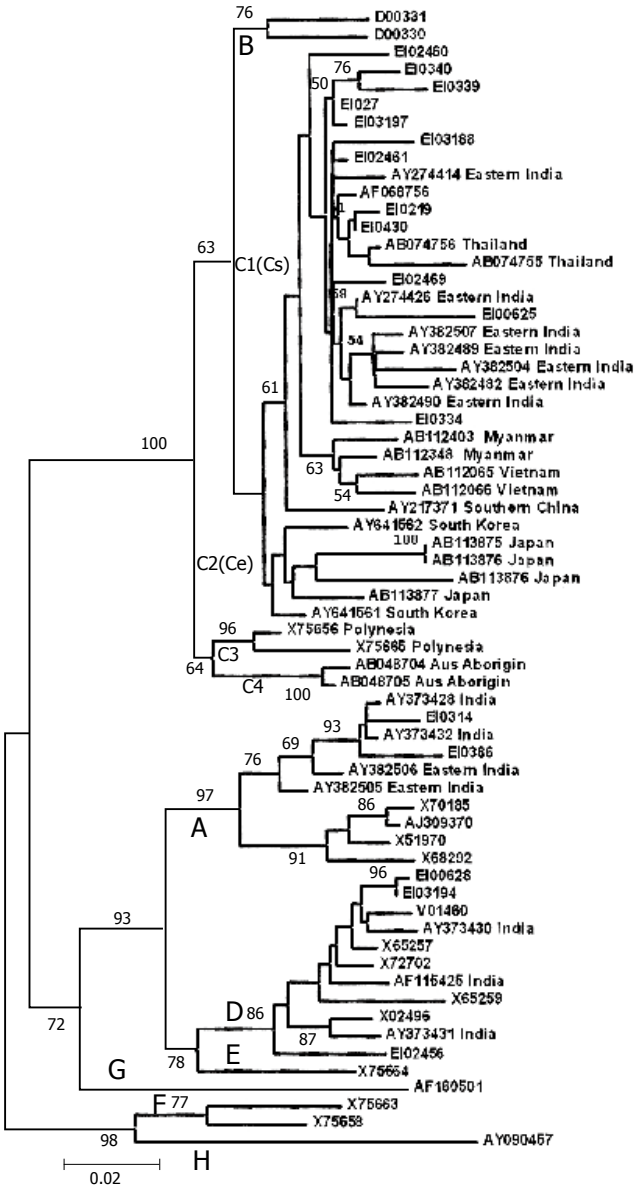

Figure 3 Neighbor joining phylogenetic analysis using the core gene region (nt. 1900-2300) of HBV isolates from Eastern India (denoted with EI).

the HBV/C strain isolated from Eastern India clustered with South East Asian strain (HBV/Cs) and also found to be most closely related to Thailand sequences.

Mutation in S gene region

Amino acid sequences of the part of S protein (codon 41-180) of 27 HBV/C isolates from our study were compared with sequences retrieved from GenBank. Subtyping of the HBV isolates was done on the basis of presence of amino acid residues at codon 122, 127 and 160 of the S gene region (Figure 4A and B). The sub determinant q+ and q- was differentiated on the basis of amino acid residue at codon 177 and 178. Among the genotype C isolates 26 out of 27 isolates had amino acid Lys122 and Arg160 as well as Val177 and Pro178 indicating that they were adrq+ subtype. However, only one isolate (EI01164) was classified as adw2 subtype on the basis of amino acid Lys122 and Lys160 and Pro127 present in the antigenic determinant.

Comparison of amino acid sequences showed that most of the sequences were conserved (Figure 4A and B). Consensus amino acid substitution at codon 53 and 126 was observed within HBV/Cs and HBV/Ce. Out of 27 genotype C sample studied 20 were shown to have Ser to Leu amino acid substitution at codon 53, which was also found in all genotype HBV/Cs sequence from the database. The remaining 7 samples had Ser at 53 codon, which was the characteristic of genotype Ce. Most of the samples from Far East (HBV/Ce) had amino acid Ile at

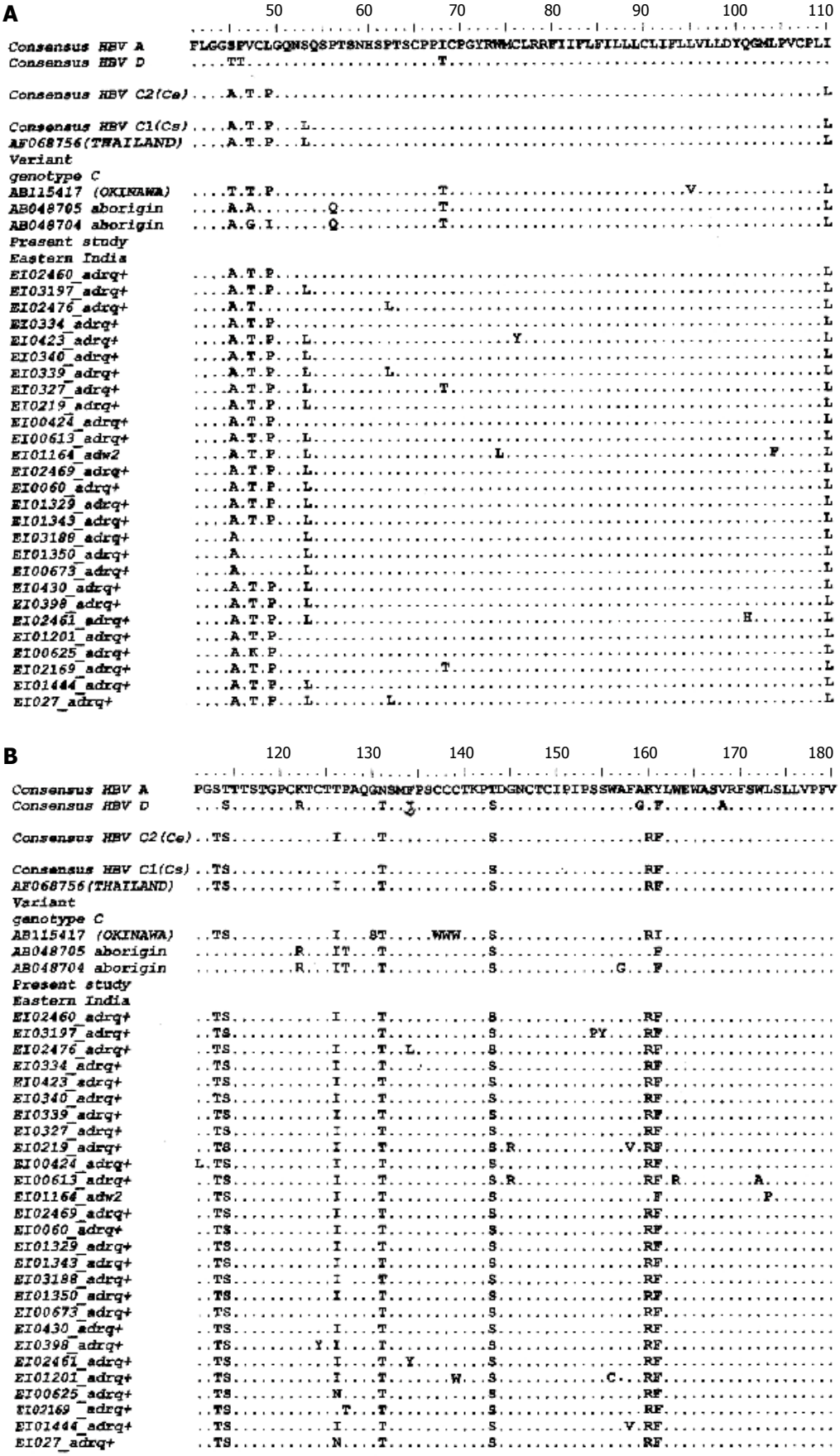

Figure 4 **A**, **B**: Alignment of amino acid sequences (41-180) of the partial S protein of HBV/C isolated from Eastern India.

codon 126, whereas most of the samples from Southeast Asia had either Asn or Thr at codon 126. However the isolate AF068756 from Thailand had Ile at codon 126. Majority of the genotype C isolates studied by us also had Ile at codon 126. Only three isolates had Thr and two had Asn in that position.

DISCUSSION

Except for the HBV sequences from two studies that focused on Western and Northern India[14,15], there are hardly any HBV sequences available from our vast country. In this study, HBV DNA sequencing and phylogenetic analysis of S gene and core region established the presence of genotype C among the HBV carriers in addition to HBV/A and HBV/D from Eastern India. This is in contrast to Western and Northern India where genotypes D and A are prevalent[14,15]. Moreover, genotype C could be detected both from Kolkata as well as from the incidentally detected asymptomatic carriers from the rural population. Vivekanandan *et al* 2004 reported the presence of genotype C (by RFLP) only among chronic patients who went to their hospital for treatment from Eastern India. However, they could not detect HBV/C among their asymptomatic relatives who were incidentally detected HBsAg positive during blood donation. On the other hand, HBV genotypes found among patients who came from Southern India to that hospital were of genotype D excepting one case. However they have not characterized the molecular features of the genotype C strains of Eastern India. Taking into consideration their report, our findings, as well as other reports from Western and Northern India, it seemed likely that genotype C is at present confined mostly to Eastern India. Similar significant difference in the geographic distribution of HBV genotypes was recently reported from Japan and USA[24,25].

Four subtypes adrq-, ayr, adw, adrq+ were associated with genotype C. Recently, association of serotype ayw3 was also found with genotype C variant, among Australian aborigines[9]. In our study, in addition to adrq+ subtype, one (EI01164) of the genotype C HBV isolates showed adw2 subtype. High prevalence of this adw2 subtype within genotype C was already reported from Tibet, East Asia[26] which borders Northern India. Genotypes A and D in our study group were of adw2 and ayw2, ayw3 subtype respectively which is consistent with the previous study reported from other parts of India[14,15].

In places where well-known waves of migration have occurred over time, prevalence of HBV genotypes is known to reflect anthropological history of human migration, origin of immigrants and other patterns of migration. Thus, an apparent south-to-north gradient of genotypes C and B in Japan is considered to reflect anthropological history of migration from Asian countries to Japan[24]. In contrast, the presence of genotype D HBV among primitive tribes of Andaman and Nicober islands is thought to be due to introduction of HBV from mainland India in the past century[27]. However, carrying the HBV/Cs strain among Jarwas (an isolated tribe from Andaman and Nicober islands) correlate with the anthropological history of migration of this tribes from Southeast Asia[28].

Population groups of Northern, Western and Eastern India ethnically are of Caucasoid origin, who speak Indo-European languages and show close genetic affinities with populations of Eurasia and Europe[29] where HBV genotypes A and D are prevalent. It is therefore not surprising that HBV genotype D and A is most predominant in India. On the other hand genotype C is predominant in countries neighboring Eastern part of India, where the population groups (of Mongoloid origin) are believed to have originated from Tibeto-Burman language subfamily. Genetic study showed that the Bengali population group of Eastern India had close ethnic affiliation with Caucasoids[30] and formed clusters distinctly different from the North East population groups (ethnic affiliation to Mongoloid) who are possibly descendents of ancestral population of China, where genotype C and B are prevalent. Since our study population is not of Mongoloid origin, therefore it is quite unlikely that presence of genotype C in Eastern India reflects the history of population migration long ago.

This is further supported from the fact that when the sequences from the present study were compared to GenBank sequences in a BLAST search, the best matches and the high-scoring matches were from Southeast Asian Countries, especially from Thailand and not from China. Phylogenetic analysis both from Surface and core gene also revealed that almost all sequences from our study group clustered with Southeast Asian HBV/Cs subgroup and not with HBV/Ce found in East Asia and predominant in China. Of the 27 genotype C isolates sequenced, 24 (90%) had percent nucleotide identity (PNI) of 99.5% ± 0.4%, with Thai genotype C sequence (Accession No. AF068756), in the S gene region.

There are several reports available where low genetic diversity of HBV strains has been considered to suggest limited and relatively recent spread of the strain over the geographical region. Arankelle *et al*[27] reported a recent introduction of the virus, on the basis of low genetic diversity of partial S gene (PNI varied from 1.6% to 2.0%), of genotype D isolates from mainland India to tribal population of Andaman and Nicobar islands. For similar reason, HBV/E was considered as relatively recent introduction in West Africa[31]. Thus considering low genetic diversity of HBV/C strain in Eastern India with Thai sequences, we presumed that the genotype Cs in Eastern India possibly might have spread from Southeast Asia, particularly from Thailand, rather recently. It is noteworthy that the prevalence of genotype C is much higher in urban population (20% *vs* 6.7%) than in rural population. Moreover, genotype C HBV samples have not been reported from Northern and Western parts of India[14,15]. In the Southern Indian Tertiary hospital[18], genotype C was detected in a significantly higher proportion of patients from Eastern India compared with those from Southern India (16.8% *vs* 0.9%, $P < 0.0001$). This selective confinement of genotype C to Eastern India especially to the urban population suggests that perhaps sufficient time has not yet passed since its introduction to Eastern India for its subsequent spread to the rest of the country. However, we must admit that this introduction of genotype C in Eastern India would have been best addressed if sequential study showing changing HBV genotype pattern could have been documented in Eastern India.

Kolkata is the most important port city of Eastern India and proximal to Southeast Asia. Increased trade relationship with Thailand suggests that spread of genotype Cs to Eastern India is possible, but the infectious source and the route of transmission are not clear. Previous reports showed presence of Thai HIV strains as well as Thai

HCV strains[32,33] from North Eastern and Eastern India. Heroin trafficking routes have been associated with IDU and HIV infection in Thailand, Myanmar, China and India[34]. HBV and HIV share common modes of transmission. There is possibility of co transmission of HBV from Southeast Asia to Eastern India, *via* this route. This might have been followed by spread to general population via unsafe injection practices, which is common in developing countries including India[35,36]. The presence of HBV/Cs is known in our neighboring country Bangladesh, however the proportion of HBV/C among HBV carriers in Bangladesh, is unknown[10]. People from Bangladesh come to Kolkata for treatment. This population might also have some influence in the presence of genotype C in Kolkata. Whatever may be the source, this study confirmed the presence of Cs in the Eastern Indian population.

In an era of frequent international travel and human migration, introduction of new HBV genotype to a community might have far reaching effects, including recombination between genotypes[37] or replacement of one genotype by another[38]. HBV genotype C is associated with delayed hepatitis B e antigen (HBeAg) seroconversion[39], more-active hepatitis[40], lower response to antiviral therapy[41], more advanced liver disease and a higher risk of hepatocellular carcinoma[42], compared with HBV genotype B. Furthermore, HBV/C with T1762/A1764 mutation in BCP region has been reported to be an increased risk factor for hepatocellular carcinoma[13]. In our study, at least 45% of the subjects were in the age group of 16-30 years. In addition, 29/42 (69%) of genotype C isolates had T1762/A1764 mutation in BCP region, most of them associated with elevated ALT. Thus, the patients infected with genotype C need to be carefully monitored to assess their clinical outcome in future.

In conclusion, the present study reports the genotypic distribution and partial genome sequences of HBV/C isolates from Eastern India in addition to most predominant HBV/A and HBV/D. These isolates clustering with HBV/Cs genotype found in South East Asia, is prevalent in considerable proportion (18%) of Eastern Indian HBV carriers. Low genetic diversity and confinement of HBV/C in Eastern India possibly indicates a recent, limited, spread of HBV/Cs in this region. Several studies have suggested that HBV genotype C is associated with more active or severe sequelae of liver disease in Southeast Asia compared with genotype B. Therefore, the presence of HBV genotype C in Eastern India should be carefully monitored by further studies including epidemiological, clinical and virological assessment.

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H pylori stimulates proliferation of gastric cancer cells through activating mitogen-activated protein kinase cascade

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Abstract

AIM: To explore the mechanism by which *H pylori* causes activation of gastric epithelial cells.

METHODS: A VacA (+) and CagA (+) standard *H pylori* line NCTC 11637 and a human gastric adenocarcinoma derived gastric epithelial cell line BGC-823 were applied in the study. MTT assay and ^{3}H-TdR incorporation test were used to detect the proliferation of BGC-823 cells and Western blotting was used to detect the activity and existence of related proteins.

RESULTS: Incubation with *H pylori* extract increased the proliferation of gastric epithelial cells, reflected by both live cell number and DNA synthesis rate. The activity of extracellular signal-regulated protein kinase (ERK) signal transduction cascade increased within 20 min after incubation with *H pylori* extract and appeared to be a sustained event. MAPK/ERK kinase (MEK) inhibitor PD98059 abolished the action of *H pylori* extract on both ERK activity and cell proliferation. Incubation with *H pylori* extract increased c-Fos expression and SRE-dependent gene expression. *H pylori* extract caused phosphorylation of several proteins including a protein with molecular size of 97.4 kDa and tyrosine kinase inhibitor genistein inhibited the activation of ERK and the proliferation of cells caused by *H pylori* extract.

CONCLUSION: Biologically active elements in *H pylori* extract cause proliferation of gastric epithelial cells through activating tyrosine kinase and ERK signal transduction cascade.

Key words: *H pylori*; Gastric cancer cells; Proliferation; Mitogen-activated protein kinase

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INTRODUCTION

H pylori is an important pathogen associated with gastritis and peptic ulcers[1]. It has also been defined as a carcinogen[2]. The mechanisms of pathogenic and carcinogenic effects of *H pylori* infection are under intensive investigation. Research data suggest that *H pylori* might stimulate the proliferation of gastric epithelial cells both *in vitro* and *in vivo*[3-5]. This effect of *H pylori* has an important pathogenic significance because increased cell proliferation may elevate the gastric mutation rate and is a predisposing factor for neoplasia. The mechanism, especially the intracellular signal transduction pathway associated with the stimulating effect of *H pylori* on the proliferation of gastric epithelial cells, is under extensive study.

Protein kinases, which regulate the protein phosphorylation, are considered to play the most important role in regulating protein function and cell activity. Mitogen-activated protein kinase (also known as MAP Kinase) is a crucial member of the protein kinase family. So far, four MAP Kinase cascades have been characterized in mammalian cells. Among them, extracellular signal-regulated protein kinase (ERK) cascade is most well characterized. The activation of signal transduction pathways by growth factors, hormones and neurotransmitters is mediated through ERK cascade[6,7]. It is the basic signal transduction pathway regulating cell proliferation and differentiation. Recent research data indicate that activation of ERK cascade is involved in *H pylori*- induced proliferation of gastric epithelial cells[8-10]. However, more details of *H pylori*-induced activation of ERK cascade are needed to be explored.

MATERIALS AND METHODS

Materials

Human gastric epithelial cell line BGC-823 (ICLC

HTL98007) was provided by Institute of Tumor Research of Beijing. VacA (+) and CagA (+) international standard *H pylori* strain NCTC11637 was provided by Institute of Microbiology and Epidemiology of China, Beijing. DMEM culture medium was from GiBco (Grand Island, NY). Brucella broth was from Becton Dickinson (Franklin Lakes, NJ). New born calf serum (NBCS) was from No.2 Factory of Beijing Milk Company (Beijing, China). Antibody against phosphorylated ERK was from Sigma (St. Louis, MO). All other primary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). HRP-conjugated secondary antibody was from Jackson ImmunoResearch Laboratories (West Grove, PA). ECL chemoluminescence reagents were from Amersham (Buckinghamshire, England). Tyrosine kinase inhibitors genistein and luciferin were from Sigma (St. Louis, MO). MAPK/ERK inhibitor PD98059 was from Calbiochem (San Diego, CA). Plasmid DNA constructs encoding SRE-luciferase and CRE-luciferase were kind gifts from Dr. Renate Pilz in University of California, San Diego.

Cell culture

Human gastric cancer cell line BGC-823 was kept in DMEM containing 100 mL/L serum and incubated in 5% CO_2 at 37℃. The medium was changed every second day and the cells were sub-cultured at 80%-90% confluence.

Preparation of H pylori extract

Healthy *H pylori* was scraped down from horse serum agar plate and transferred into Brucella broth containing 100 mL/L serum. The bacterial suspension was incubated in micro-oxygen environment (80% N_2, 15% CO_2, 5% O_2) at 37℃ for 24 h with continuous shaking. The growth of *H pylori* was confirmed by Gram staining before harvest. *H pylori* cells were precipitated by centrifugation at 5000 r/min for 15 min at 4℃ and washed with PBS. The bacteria were re-suspended in serum free cell culture medium (DMEM) and sonicated (100 W, 15 s × 6, at 30 s interval). The breakdown of the bacteria was confirmed by microscopy. The suspension was centrifuged at 10000 r/min for 30 min at 4℃. The supernatant (*H pylori* extract) was adjusted to protein concentration of 1 g/L and kept at -20℃ until use.

Preparation of protein samples

The harvested cells (1×10^7) were washed three times with PBS and re-suspended in 40 μL suspension buffer containing 100 mmol/L NaCl, 10 mmol/L Tris-HCl (pH7.6), 1 mmol/L EDTA (pH8.0), 1 mg/L aprotinin, 100 mg/L PMSF. The same volume of boiled 2 × SDS protein loading buffer containing 100 mmol/L Tris-HCl (pH6.8), 200 mmol/L DTT, 4% SDS, 0.2% bromophenol blue, 20% glycerol was added into the cell suspension. The sample was boiled for 10 min to lyse the cells and sonicated for 1 min to break down the DNA. After centrifugation at 12000 r/min for 10 min, the supernatant was kept at -20℃ until use. For preparation of membrane protein samples, the cells were harvested, washed with PBS, and lysed with extract buffer containing 20 mmol/L HEPES (pH7.4), 10 mL/L Triton X-100, 5 mmol/L EDTA, 50 mmol/L NaCl, 30 μmol/L β-glycerophosphate, 50 mmol/L sodium fluoride, 50 mg/L aprotinin, 10 mg/L leupeptin. The lysate was centrifuged at 100000 × *g* for 1 h at 4℃ and the supernatant was kept at -20℃ until use.

Western blotting

The protein sample was loaded with 30 μg protein per lane on SDS-PAGE gel. After electrophoresis, the proteins on the gel were transferred onto nitrocellulose (NC) membrane as described previously. The NC membrane was blocked with 50 g/L milk in TBS-T for 1 h at room temperature (RT), incubated with first antibody for 2 h and with secondary antibody for 50 min at RT and finally washed three times after each incubation. ECL chemoluminescence reagent was used to show the positive bands on the membrane.

MTT assay

A total of 1×10^4 trypsin-dispersed BGC-823 cells in 0.1 mL culture medium were seeded into each well of the 96-well plates and cultured for 24 h. Then, the cells were incubated with medium alone or with medium plus *H pylori* extract at different concentrations. After 24 h of further incubation, 10 μL of MTT (6 g/L, Sigma) was added to each well and the incubation was continued for 4 h at 37℃. Finally, the culture medium was removed and 200 μL of dimethylsulphoxide (DMSO) was added to each well. The absorbance was determined with an ELISA reader at 570 nm. The "change percentage" of the absorbency (*A*) value of the cells treated with *H pylori* extract compared with *A* value of the cells without treatment was calculated as:

$$\text{Change (\%)} = \frac{A \text{ value } (H \textit{ pylori} \text{ group-control})}{A \text{ value of control group}} \times 100\%$$

^{3}H-TdR incorporation

The cells were cultured in the same way as described in MTT assay. When the culture medium was changed and *H pylori* extract was added, 0.2 μCi of ^{3}H-TdR was also added into each well. After further incubation for 24 h, the medium was discarded and the cells were trypsin-dispersed and collected onto a membrane. The membrane was heated dry at 65℃ and put into a plastic vial containing 6 mL of scintillation liquid. The count per minute (CPM) was calculated with a Beckman scintillation counter. The "change percentage" of CPM of the cells treated with *H pylori* extract compared with CPM of the cells not treated with *H pylori* extract was calculated as:

$$\text{Change (\%)} = \frac{\text{CPM } (H \textit{ pylori} \text{ group-control})}{\text{CPM of control group}} \times 100\%$$

Reporter gene assay

The cells were transfected with plasmid DNA encoding SRE-luciferase or CRE-luciferase, and β-galactosidase. After incubation with *H pylori* extract for 8 h, the cells were harvested and the activity of luciferase and β-galactosidase was measured by luminescence-based assays. The luciferase activity was calculated as the fold of increase after normalized by the β-galactosidase activity.

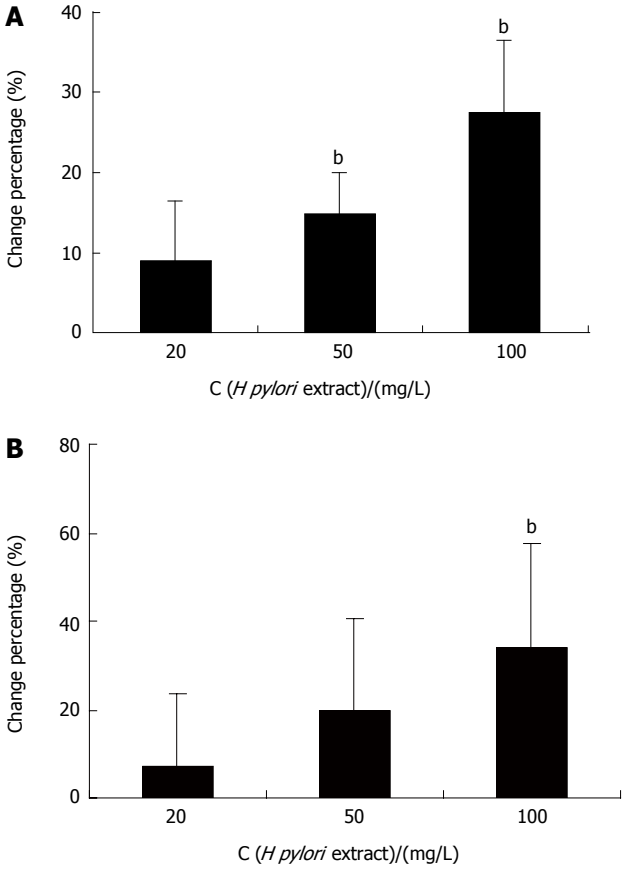

Figure 1 MTT assay (**A**) and ^{3}H-TdR-incorporation test (**B**) showing *H pylori* extract- stimulated proliferation of BGC-823 cells. $^bP < 0.01$ *vs* control.

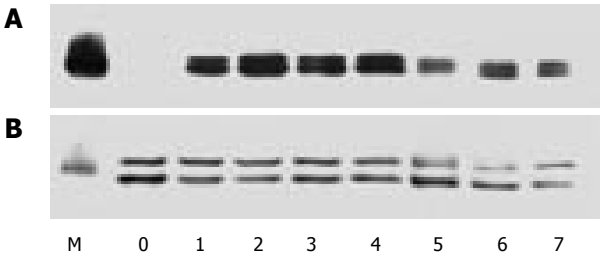

Figure 2 Western blotting showing phosphorylated ERK (**A**) and total ERK (**B**) in serum-starved BGC-823 cells incubated with H pylori extract. M: Protein molecular marker; 0: Control; 1-7: Incubation with 50 mg/L *H pylori* extract for 20, 40, 60 min and 3, 6, 12, 24 h.

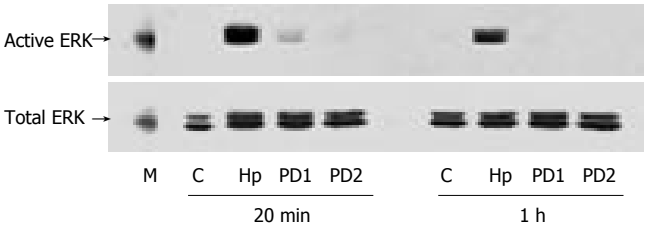

Figure 3 Western blotting showing PD98059-blocked stimulating effect of *H pylori* extract on ERK activation in BGC-823 cells. M: Molecular marker; C: Control; Hp: 50 mg/L *H pylori* extract; PD1: 25 μmol/L PD98059 + 50 mg/L *H pylori* extract; PD2: 50 μmol/L PD98059 + 50 mg/L *H pylori* extract.

Statistical analysis

The results of MTT assay, ^{3}H-TdR incorporation test and reporter gene assay data were expressed as means ± SD. The significance of the difference between control group and experimental group was evaluated by Student's *t*-test.

RESULTS

H pylori extract stimulated proliferation of BGC-823 cells

The MTT assay showed that *H pylori* extract could stimulate the proliferation of BGC-823 cells. The *A* value reflecting the number of viable cells, increased significantly in the cells incubated with 50 mg/L and 100 mg/L *H pylori* extract for 24 h (Figure 1A). Similar result was also obtained in ^{3}H-TdR-incorporation test. The ^{3}H incorporation increased dose-dependently when the cells were incubated with 20 mg/L, 50 mg/L, and 100 mg/L *H pylori* extract respectively for 24 h (Figure 1B).

H pylori extract activated extracellular signal-regulated kinase in BGC-823 cells

The separated protein samples from cells incubated with 50 mg/L *H pylori* extract for different periods of time were first probed with antibody against phosphorylated ERK to analyze the activity of ERK and re-probed with anti-ERK2 antibody to show the protein content of this kinase. The results showed that in serum-starved cells, the activity level of ERK was very low. During incubation with *H pylori* extract, the activity of ERK increased obviously within 20 min and stayed at a high level (about 5 times of control) for more than 8 h (Figure 2).

PD98059 blocked the stimulating effect of H pylori extract on ERK activity and proliferation of BGC-823 cells

PD98059, an inhibitor of MAPK/ERK kinase (MEK) activating ERK directly, was used to confirm the specificity of ERK activation induced by *H pylori* extract. Western blotting showed that PD98059 abolished the stimulating effect of *H pylori* extract on ERK activity (Figure 3). MTT assay showed that the inhibitor also blocked the stimulating effect of *H pylori* extract on proliferation of the cells (Figure 4).

Genistein inhibited ERK activation induced by H pylori extract

Genistein is an inhibitor of tyrosine kinase. Western blotting with antibody against phosphorylated ERK showed that treating the cells with genistein inhibited the ERK activation induced by *H pylori* extract (Figure 5).

H pylori extract caused tyrosine phosphorylation of membrane protein

An antibody against pan-tyrosine phosphorylation of proteins was used to detect the tyrosine phosphorylation caused by *H pylori* extract. Western blotting showed that during incubation with *H pylori* extract, several proteins were phosphorylated on tyrosine within 20 min of incubation (Figure 6). A tyrosine-phosphorylated protein with molecular size around 97.4 kDa was especially arrested because it also existed in membrane protein sample extracted from the cells (Data not shown).

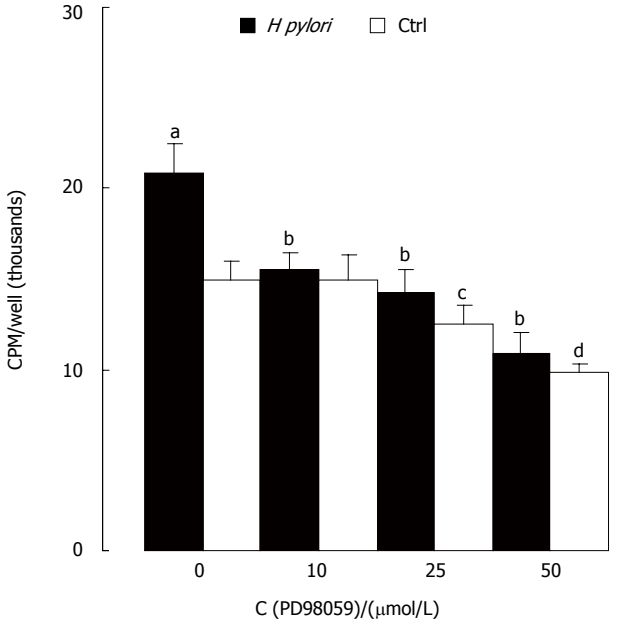

Figure 4 MTT assay showing PD98059-blocked proliferation-stimulating effect of H pylori extract. $^{a}P < 0.05$, $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* control only; $^{b}P < 0.01$ *vs* Hp only.

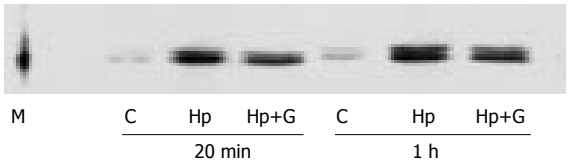

Figure 5 Western blotting showing genistein-prevented ERK activation by *H pylori* extract. M: Molecular marker; C: Control; Hp: *H pylori* extract; Hp + G: Genistein and *H pylori* extract.

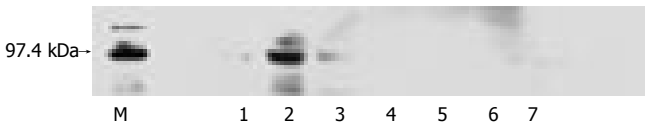

Figure 6 Western blotting showing *H pylori* extract-caused tyrosine phosphorylation of cell lysate of BGC-823. M: Molecular marker; 1: control; 2-7: *H pylori* extract incubated for 20, 40 min and 1, 3, 6, 12 h.

H pylori extract increased expression of c-fos

Western blotting showed that when the cells were incubated with 50 mg/L *H pylori* extract, the expression of c-Fos protein increased obviously. During incubation, the expression of c-Fos started to increase around 40 min after incubation, reached its peak around 1 h and lasted for 6 h (Figure 7).

H pylori extract stimulated SRE-dependent reporter gene expression

Reporter gene assay showed that *H pylori* extract specifically stimulated SRE-dependent expression of luciferase while it had no effect on CRE-driven luciferase expression (Figure 8), indicating that *H pylori* extract could increase c-Fos expression through SRE cis-element of the promoter.

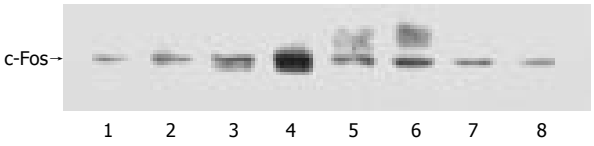

Figure 7 Western blotting showing *H pylori* extract- increased expression of c-Fos. 1: Control; 2-8: *H pylori* extract incubated for 20, 40 min and 1, 3, 6, 12 and 24 h.

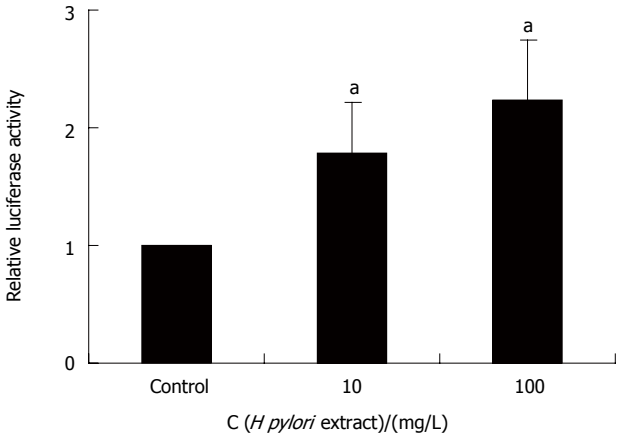

Figure 8 *H pylori* extract-stimulated SRE-dependent reporter gene expression. $^{a}P < 0.05$ *vs* control.

DISCUSSION

Gastric *H pylori* infection is the most common infection in humans. It is responsible for virtually all cases of gastritis and most cases of peptic ulcers[1]. Moreover, *H pylori* is defined as a group 1 carcinogen in humans by International Agency for Research on Cancer, a working party of WHO[2]. The conclusion is, however, mainly based on the epidemiological data and clinical investigations. Even though laboratory data on carcinogenesis of *H pylori* are accumulating during recent years[11-13], more efforts are still needed to elucidate the mechanism of *H pylori*-induced malignant diseases. Gastric carcinoma develops through a sequence of events from normal mucosa to gastric carcinoma[14]. *H pylori* might be closely associated with this process. Several mechanisms have been proposed by which *H pylori* infection might lead to predisposition for gastric cancer[15]. One explanation is that *H pylori*-associated chronic inflammation may increase the rate of cell turnover in gastric mucosa. Rapidly replicating DNA is more susceptible to damage. Alongside increased reactive oxygen species and decreased ascorbic acid content during inflammatory reactions, *H pylori* -induced proliferation may be the major factor for gastric dysplasia and gastric cancer[16,17]. In *H pylori* hosts with gastritis, increased proliferation of gastric epithelial cells has been confirmed[3,4]. Laboratory data have also shown that *H pylori* might directly stimulate proliferation of gastric epithelial cells[5,10,11]. The former reveals an increased cell turnover rate in *H pylori* host and the latter suggests that increased proliferation of gastric epithelial cells might be a direct effect of *H pylori*.

Probing separated proteins with anti-phosphorylated ERK antibody and re-probing them with anti-ERK2

antibody provide a sensitive way to detect ERK activity[18]. With this method, our study showed that during incubation with *H pylori* extract, the activity of ERK increased obviously in serum-starved cells and the activation of ERK was a sustained event. This is significant because sustained activation of ERK appears to be required for many cells to pass the gastrointestinal restriction point to enter S phase, in which cellular DNA is replicated[19,20]. MTT assay and ^{3}H-TdR-incorporation test confirmed the stimulating effect of *H pylori* extract on cell proliferation in our study. Since ERK-mediated cascade is the basic signal transduction pathway regulating cell proliferation, this pathway can mediate proliferation-stimulating effects of *H pylori*.

ERK is in the middle of the signal transduction pathway. Currently, it is believed that ERK may be activated through several pathways[21]. One is the receptor tyrosine kinase-Ras-MEK pathway. Growth factor ligand binds to membrane receptor and causes tyrosine-phosphorylation of the receptor. Consequential action of Ras and then Raf, which are components of the cascade, causes the activation of MEK (also known as MAP kinase), a direct upstream regulator of ERK[22]. PD98059 is a specific inhibitor of MEK[23]. Our results showed that PD98059 could block both ERK activity-stimulating and proliferation-stimulating effects of *H pylori* extract. Ras activity was not investigated in this experiment. However, we used tyrosine kinase inhibitor genistein to explore the possible involvement of tyrosine kinase in *H pylori* extract-induced signaling. The results showed that genistein inhibited *H pylori* extract-induced activation of ERK. Then, we used antibody against pan-tyrosine phosphorylation to detect tyrosine phosphorylation in both whole cell lysate and membrane protein from the cells. The results showed that in samples from the cells treated with *H pylori* extract, there was obvious tyrosine phosphorylation of a membrane protein with molecular size around 97.4 kDa, suggesting that it is very likely that *H pylori* extract activates ERK through receptor tyrosine kinase-Ras-MEK pathway.

As to the down-stream events of ERK activation, it was reported that some ERK activated by MEK can translocate to the nuclei and phosphorylate at least two transcription factors, c-Myc and Elk-1[24,25]. The phosphorylated factors may lead to increased production of c-Fos mRNA and finally lead to cell activation[26]. Mitsuno *et al*[27] reported that co-culturing human gastric cancer cells with Cag-positive *H pylori* strains can activate c-fos gene through SRE. Our results showed that *H pylori* extract stimulated SRE-dependent gene transcription and increased the expression of c-Fos protein, indicating that the signaling process behind activation of ERK by *H pylori* can derive biological elements.

Several methods, including co-culture of live *H pylori* with target cells, use of water extract and sonication extract to stimulate target cells, have been used to study the effect of biologically active components of *H pylori* on the activity of target cells. For example, during co-culture, *H pylori* may secrete active factors or act on the target cells through type IV secretion system[28], water extracting can get the surface component of the bacteria[29], and sonication extracting can efficiently release active components from the body of bacterial cells[30,31]. In this experiment, sonication of *H pylori* extract showed that soluble component from the bacteria activated the MAPK cascade of gastric cells. Further work is needed to identify the molecular features of the component in our laboratory.

In summary, *H pylori* extract has direct proliferation-stimulating effects on gastric epithelial cells and the activation of gastric epithelial cells can be induced by *H pylori* through MAPK-mediated signal transduction pathway, suggesting that biologically active elements in *H pylori* contribute to the tumorigenesis effect of the bacteria.

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BASIC RESEARCH

Lactobacilli, bifidobacteria and *E. coli* nissle induce pro- and anti-inflammatory cytokines in peripheral blood mononuclear cells

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Abstract

AIM: To investigate whether the stimulation of peripheral blood mononuclear cells (PBMNC) with the cell debris and cell extraction of different probiotic strains is similar or species specific.

METHODS: Three strains of *bifidobacteria*, 4 strains of *lactobacilli*, and *E. coli* nissle were sonicated and centrifuged in order to divide them into cell extract and cell debris. PBMNC were separated by density gradient and incubated for 36 h with either the cell debris or the cell extract of single strains of probiotic bacteria in doses from 10^2 to 10^8 CFU/mL. Cell supernatants were taken and interleukin (IL)-10, IL-1β, and tumor necosis factor (TNF)-α were determined by ELISA.

RESULTS: Depending on the species super-family, the strains had different stimulation patterns. Except for both L. casei strains, the cell extract of *bifidobacteria* and *lactobacilli* had less stimulating capacity than cell debris, whereas the cell extract of *E. coli* nissle had similar stimulating properties to that of the cell debris of the strain and significantly more stimulating capacity than that of *bifidobacteria* and *lactobacilli*. The cell debris of *bifidobacteria* stimulated more cytokine release than the cell debris of *lactobacilli*. The cell debris of *lactobacilli* did not have a stimulating capacity when lower concentrations were used. Neither cell extraction nor cell debris had an inhibitory effect on the production of the tested cytokines by stimulated PBMNC.

CONCLUSION: The incubation of probiotic strains, which have been used in clinical trials for inflammatory diseases, with immunocompetent cells leads to different species specific reactions. High IL-10 response to cell debris of *bifidobacteria* and *E. coli* nissle can be found. This corresponds to positive effects of *bifidobacteria* and *E. coli* nissle in clinical trials for inflammatory bowel disease compared to negative outcomes obtained with *lactobacilli*.

Key words: *Lactobacilli*; *Bifidobacteria*; Probiotics; Interleukin-10; Tumor necrosis factor-α; Interleukin-1β; Peripheral blood mononuclear cells

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INTRODUCTION

There is no doubt that the relationship between intestinal microflora and immune system is complex. The host has to distinguish between pathogenic bacteria and harmless commensal and must react in an adequate and not self-destructive manner[1]. There is an increasing body of evidence that chronic intestinal inflammations such as

inflammatory bowel disease (IBD) are due to a disturbed relationship within the host's immune response to the enteric microflora[2-4]. Based on these proposals the relationship between micro-flora and intestinal immune response has been intensively studied by manipulation of the enteric micro-flora with probiotic bacteria[5-11]. These are by definition "A preparation of or a product containing viable, defined products in sufficient numbers which alter the microflora (by implantation or colonization) in a compartment of the host and by exerting beneficial health effects in the host"[12]. The efficacy of *E. coli* nissle in therapy for ulcerative colitis[5-6] has been shown and our own clinical experience has been focused on a highly concentrated probiotic preparation (VSL#3) in preventing pouchitis, an unspecific inflammation of an ileal pouch anal anastomosis after colectomy for ulcerative colitis[9-10]. *Lactobacillus* GG instead has no influence on clinical outcome in Crohn's disease[7-8]. Besides clinical findings, different studies have also shown the influence of probiotic bacteria on the local and systemic immune response in experimental colitis[13-15]. The mechanisms underlying this effect are still under investigation. One hypothesis is based on modulating pro- and anti-inflammatory cytokines[16-17]. Pro-inflammatory cytokines such as interleukin (IL)-1β and tumor necosis factor (TNF)-α play an important role in gut inflammation[17]. In Crohn's disease and ulcerative colitis these cytokines are elevated in local inflammation area and peripheral blood cells[18-19], whereas the anti-inflammatory cytokine IL-10 is decreased in patients suffering from IBD[20]. IL-10 produced by gene manipulated bacteria, reduces toxic colitis that is associated with pro-inflammatory cytokines[21-22]. Further arguments have been found in the absence of IL-10. IL-10 deficient (KO) mice do not show any symptoms of intestinal inflammation as long as they are kept in sterile conditions, but spontaneously develop chronic colitis with a histological distribution similar to that found in Crohn's disease after termination of the sterile conditions[23]. If the mice are fed with different lactic acid bacteria before finishing the sterile conditions, this chronic inflammation can be prevented[24]. Recently, during preventive therapy for chronic pouchitis using different probiotics as mentioned above, we investigated the cytokine tissue levels of patients with pouchitis and after induction of remission and during the following probiotic application. It is interesting to find that cytokine tissue levels of IL-10 increase during the application of the probiotic preparation, whereas anti-inflammatory cytokine-levels such as TNF-α and IL-1 remain low after the application[25].

The aim of our study was to set up an *in vitro* model to compare the immunomodulatory effects of different probiotic strains that have previously been evaluated in different clinical trials. For this purpose we used peripheral blood mononuclear cells (PBMNC), which are a combination of different immunogenic cells.

MATERIALS AND METHODS

Subjects

Blood samples were taken from 12 healthy blood donors (7 females, mean age: 44 years; 5 males, mean age: 52 years). Blood from the same donor was used for each co-incubation with all tested bacteria in different concentrations. Incubation experiments were repeated 4 to 6 times with blood from different donors. The study was performed in accordance with the Declaration of Helsinki and the local ethics committee.

Table 1 List of the bacterial strains

Bifidobacterium breve: **Y 8**
Bifidobacterium infantis: **Y 1**
Bifidobacterium longum: **Y 10**
E. coli: Stamm Nissle 1917 (Mutaflor, Ardeypharm, Herdecke, Germany)
Lactobacillus azidophilus: **MB 443**
Lactobacillus casei subspecies *rhamnosus*: Lactobacillus GG (LGG, Valio, Helsinki, Finland): ATCC 53103
Lactobacillus casei: **MB 451**
Lactobacillus delbrueckii subspecies *bulgaricus*: **MB 453**
Lactobacillus plantarum: **MB 452**

Separation of PBMNC

PBMNC from healthy donors were separated according to Boyum[26]. Briefly, peripheral blood diluted with Hank's balanced salt solution (HBSS) (without Ca^{2+} or Mg^{2+}) (GIBCO, Karlsruhe, Germany) containing 100 U/mL heparin, was layered over a ficoll (Lymphoprep, Progen, Biotechnik, Heidelberg, Germany; specific gravity: 1.077) and centrifuged for 40 min at 400 r/min without using a frame. Cells harvested from the interface were washed in HBSS (without Ca^{2+} or Mg^{2+}) and centrifuged for 10 min at 400 r/min. Supernatant was discarded and the pellet was resuspended in HBSS (without Ca^{2+} or Mg^{2+}), which was repeated four times. Finally, resuspension was performed in RPMI 1640 (GIBCO, Karlsruhe, Germany) with 10% fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 50 μg/mL gentamycin (all Sigma, St. Louis, MO). Viability of cells was tested by trypan-blue, which was more than 97%. After calculation of the cells per volume, the cell count was adjusted to 500 000 cells per well and per mL.

Bacteria and culture conditions

The bacteria species and strains used in this study are listed in Table 1. Strains in bold type originated from the pharmaceutical probiotic VSL#3 (Sigma Tau, Promezia, Italy). *Bifidobacterium* and *Lactobacillus* strains were grown in MRS broth (Difco, Detroit, MI) with the addition of 0.05% L-cysteine hydrochloride monohydrate (Merck, Darmstadt, Germany). All strains were incubated anaerobically at 37℃. Mid log cultures counted by plating technique on the above mentioned media, were collected by centrifugation (8 000 × *g* for 3 min), washed and resuspended in 5 mL RPMI 1640 Medium (GIBCO, Karlsruhe, Germany). The bacterial suspensions were subsequently sonicated (Branson Sonifier W-250, Heinemann, Schwäbisch, Germany) at power levels 5-6 at 30% duty for 5 min to destroy cellular membranes. The sonicated suspension was centrifuged at 8 000 × *g* for 30 min to separate cell debris from crude cell extract. After centrifugation the supernatant containing the

cell extract and the pellet containing the cell debris were taken for further investigation.

Bacterial incubation with PBMNC

Bacterial cell debris and extract were applied at concentrations ranging from 1×10^3 to 1×10^{10} colony forming units (CFUs)/mL. One hundred μL at specific concentration was transferred to 900 μL medium containing 500 000 mononuclear cells and co-incubated at 37℃ and 50 mL/L CO_2 for 36 h. Supernatants were collected and stored at -20℃ until assay. The viability of PBMNC was checked by the trypan blue test. On each incubation plate a positive control with LPS added to PBMNC and a negative control without stimulus were investigated. The same set-up was used in order to examine the ability of probiotics to inhibit LPS-induced cytokine release. PBMNC were incubated with bacterial cell extract and debris for 10 min, then LPS was added in a concentration of 100 ng/μL.

Cytokine quantification in culture supernatant

Cytokine quantification in culture supernatant was analysed by commercially available sandwich enzyme linked immunosorbent assay (ELISA).

TNF-α was detected using anti-human TNF-α monoclonal "capture" antibody (MAB 610, R&D Systems, Minneapolis, MN, USA) and biotinylated "detection" antibody (BAF 210 R&D Systems, Minneapolis, MN, USA) with o-phenylenediamine buffer/H_2O_2 (Sigma, Steinheim, Germany) as substrate. Standard procedure was performed by using recombinant human TNF-α (210-TA, R&D Systems, Minneapolis, MN, USA). The absorbance values of the sample were read at 490 nm on an ELISA plate reader. ELISA measurements were performed in duplicates or triplicates.

IL-1β was detected using anti-human IL-1β monoclonal "capture" antibody (MAB 601, R&D Systems, Minneapolis, MN, USA) and biotinylated "detection" antibody (BAF 201 R&D Systems, Minneapolis, MN, USA) with o-phenylenediamine buffer/H_2O_2 (Sigma, Steinheim, Germany) as substrate. Standard procedure was performed by using recombinant human IL-1β (201-LB, R&D Systems, Minneapolis, MN, USA). The absorbance values of the sample were read at 490 nm on an ELISA plate reader. ELISA measurements were performed in duplicates or triplicates.

IL-10 was detected using anti-human IL-10 "capture" antibody (18551A Pharmingen, San Diego, CA, USA) and biotinylated "detection" antibody (18562D, Pharmingen, San Diego, CA, USA) with o-phenylenediamine buffer/H_2O_2 (Sigma, Steinheim, Germany) as substrate. Standard procedure was performed by using recombinant human IL-10 (19701V, Pharmingen, San Diego, CA, USA, USA). The absorbance values of the sample were read at 490 nm on an ELISA plate reader. ELISA measurements were performed in duplicates or triplicates.

Statistical analysis

Data of cytokine concentration were presented as mean ± SE and expressed in pg/mL. For quantification of stimulating capacity of bacteria at different concentrations, cytokine concentration was resumed as an area under the curve (AUC) and described as (mean ± SE) AUC. Statistical significance was calculated by the Mann-Whitney-Rank test and expressed as *P*-value.

RESULTS

Positive and negative controls

Results from cytokine production in PBMNC after stimulation with LPS (100 ng/mL) were pooled (144 samples from 12 different donors). The mean cytokine production in stimulated PBMNC was 186.5 ± 125.6 pg/mL for IL-10, 1875.6 ± 1381.2 pg/mL for IL-1β and 356.0 ± 249.1 pg/mL for TNF-α. Cytokine production in non-stimulated PBMNC was under detection limit of the ELISA.

Cytokine production in PBMNC after incubation with cell debris and extract

Generally, cytokine production in PBMNC induced by bacteria differed in cell debris and extract between bacteria and depended on the applied concentration used (Figure 1).

IL-10 concentration

***Lactobacilli*:** Cell extracts from all applied *lactobacilli* induced IL-10 concentration only weakly, whereas no difference was found between species (data not shown). The stimulation by cell debris of these strains led to higher concentrations of IL-10 whereas significance only reached in *L. azidophilus* MB443, *L. delbrueckii* subsp. *bulgaricus* MB453 and *L. plantarum* MB452 (AUC: *L. azidophilus* MB443: 1.0 ± 1.5 pg/mL; *L. delbrueckii* subsp. *bulgaricus* MB453: 3.8 ± 4.65 pg/mL; *L. plantarum* MB452: 9.9 ± 13.8 pg/mL). The stimulation by cell debris of both *L. casei* subs. (MB 451 and *L. GG*)) did not differ from that of cell extract of these strains. As shown in Figures 2 and 3, the cell debris of *lactobacilli* had a weak stimulation capacity at concentrations less than 10^5 CFU/mL.

***Bifidobacteria*:** Cell debris of each *bifidobacteria* strain stimulated IL-10 production in PBMNC more significantly than their cell extracts (AUC: *B. breve* Y8 cell debris: 1062.7 ± 889.9 pg/mL; cell extract: 182.6 ± 177.5 pg/mL; AUC *B. longum* Y10 cell debris: 682.7 ± 466.9 pg/mL; cell extract: 30.5 ± 28.9 pg/mL; AUC *B. infantis* Y1 cell debris: 1095.6 ± 925.3 pg/mL; cell extract: 228.3 ± 233.0 pg/mL; $P < 0.05$) (Figure1). No difference was found between different *bifidobacteria* species when the stimulation capacity of cell extracts or cell debris was compared. Cell debris from *bifidobacteria* stimulated IL-10 production in PBMNC more significantly than *lactobacilli* (AUC cell debris of *bifidobacteria*: 710.1 ± 795.5 pg/mL; AUC cell debris of *lactobacilli*: 219.6 ± 174.7 pg/mL; $P < 0.02$) (Figure 4).

As shown in Figure 2 and Figure 3, the cell debris of *bifidobacteria* had a weak stimulating capacity at the concentration lower than 10^3 CFU/mL. Moreover, the highest concentration (10^8 CFU/mL) of *bifidobacteria* had a less stimulating capacity than the lower concentration (10^7 CFU/mL), whereas the viability measured by trypan blue test was over 97%.

***E. coli* nissle:** Cell extracts and debris of *E. coli* nissle led

to similar IL-10 concentrations (AUC cell debris: 1270.7 ± 210.9 pg/mL; cell extract: 1154.8 ± 264.0 pg/mL) (Figure 1). The stimulation ability of cell extract to produce IL-1β in PBMNC was significantly higher in *E. coli* nissle than in *lactobacilli* or *bifidobacteria* ($P < 0.05$). The cell debris of *E. coli* nissle had a similar stimulating capacity to *bifidobacteria* but a more significant capacity than *lactobacilli* ($P < 0.02$) (Figure 4). The cell debris and extract of *E. coli* nissle even had a stimulating capacity at low concentrations (Figure 3). The highest concentration (10^8 CFU/mL) of *E. coli* nissle had a less stimulating capacity than the lower concentration (10^7 CFU/mL), whereas the viability measured by trypan blue test was over 97%.

IL-1β concentration

***Lactobacilli*:** As shown in Figure 1 cell extracts from all applied *lactobacilli* induced IL-1β concentration only weakly, whereas no difference was found between species (data not shown). The stimulation by cell debris of *L. acidophilus*, *L. delbrueckii* subsp. *bulgaricus* and *L. plantarum* led to higher concentrations of IL-1β (AUC: *L. azidophilus* MB443: 1282.2 ± 987.9pg/mL; *L. delbrueckii* subsp. *bulgaricus* MB453: 4881.5 ± 893.8 pg/mL; *L. plantarum* MB452: 3390.8 ± 288.9 pg/mL) ($P < 0.05$) (Figure 1). The stimulation by cell debris did not differ from cell extract of *L. casei* subs. *rhamnosus* (L.GG) and *L. casei* MB451. As shown in Figures 2 and 3 , the cell debris of *lactobacilli* had only a weak stimulating capacity at concentrations of less than 10^6 CFU/mL.

***Bifidobacteria*:** Cell debris of each *bifidobacteria* strain stimulated IL-1β production in PBMNC more significantly than their cell extract (AUC: *B. breve* Y8 cell debris: 11152.9 ± 2547.7 pg/mL; cell extract: 1488.3 ± 454.0 pg/mL; AUC *B. longum* Y10 cell debris: 12364.0 ± 192.5 pg/mL; cell extract: 491.0 ± 190.6 pg/mL; AUC *B. infantis* Y1 cell debris: 9018.8 ± 2190 pg/mL; cell extract: 2142.3 ± 925.0 pg/mL; $P < 0.05$) (Figure 1). No difference was found between *bifidobacteria* species when the stimulating capacity of cell extracts or cell debris was compared. The cell debris from *bifidobacteria* led to higher IL-1β concentrations in supernatant of PBMNC than cell debris of *lactobacilli* (AUC cell debris of *bifidobacteria*: 11692.8 ± 2283.2 pg/mL; AUC cell debris of *lactobacilli*: 3143.3 ± 2689.0 pg/mL; $P < 0.02$) (Figure 4). As shown in Figure 2, the cell debris of *B. breve* Y8 and *B. longum* Y10 even had a stimulating capacity at low concentrations (10^3 CFU/mL and 10^2 CFU/mL), whereas the IL-1β production in PBMNC was weak when incubated with *B. infantis* Y1 at a concentration of less than 10^4 CFU/mL. The highest cell debris concentration (10^8 CFU/mL) of *bifidobacteria* had a less stimulating ability to produce IL-1β in PBMNC than its lower concentration, whereas the viability measured by trypan blue test was over 97%.

***E. coli* nissle:** Cell extracts and debris of *E. coli* nissle led to similar IL-1β concentrations (AUC cell debris: 9334.8 ± 2486.1 pg/mL; cell extract: 7875.0 ± 1595.3 pg/mL) (Figure 1). The stimulating ability of the cell extract of *E. coli* nissle to produce IL-1β in PBMNC was significantly higher than that of *lactobacilli* or *bifidobacteria* ($P < 0.05$). The cell debris of *E. coli* nissle had a similar stimulating capacity to *bifidobacteria* but a more significant capacity than *lactobacilli* ($P < 0.02$) (Figure 4). The cell debris and extract of *E. coli* nissle even had a stimulating capacity at low concentrations (Figure 3). The highest concentration of debris of *E. coli* nissle had a less stimulatory capacity than lower concentration.

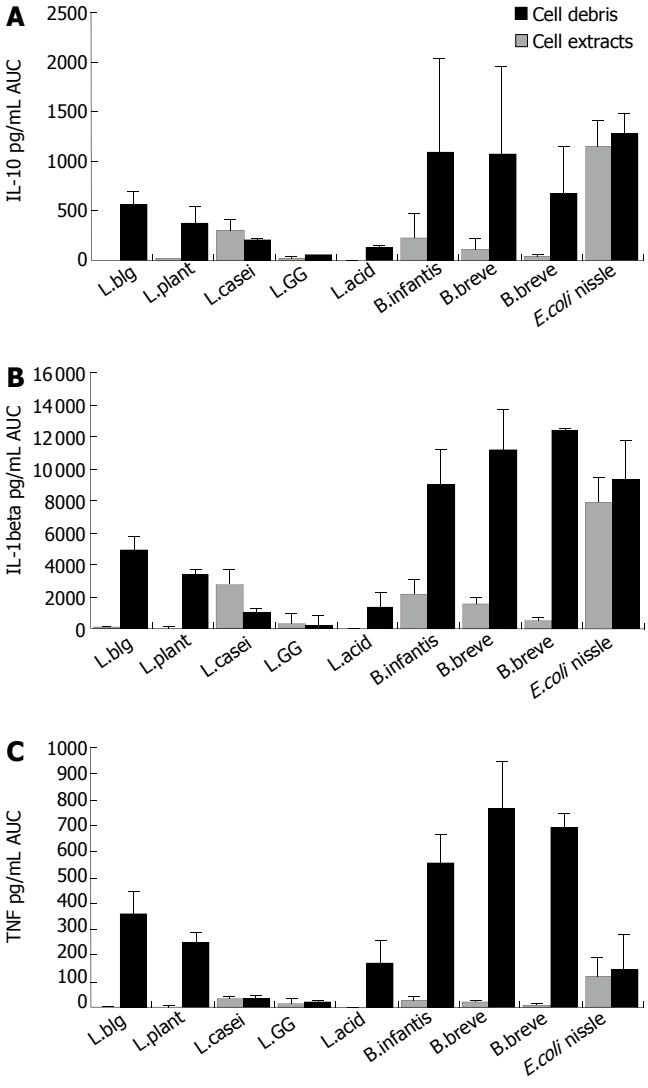

Figure 1 Concentration of IL-10 (**A**), IL-1β (**B**) and TNFα (**C**) by PBMNC (area under the curve of mean ± SE) after incubation with cell debris (■) or cell extract (▨) of different strains.

TNF-concentration

***Lactobacilli*:** Cell extracts from *L. azidophilus* MB443, *L. delbrueckii* subsp. *bulgaricus* MB453, and *L. plantarum* MB452 induced TNF-α concentration only weakly. The stimulation by cell debris of these strains led to higher TNF-α production (AUC: *L. azidophilus* MB443: 1695.3 ± 879.3 pg/mL; *L. delbrueckii* subsp. *bulgaricus* MB453: 3593.2 ± 822.1 pg/mL; *L. plantarum* MB452: 2466.5 L ± 433.3 pg/mL) ($P < 0.05$) (Figure 1). The stimulation by cell debris did not differ from cell extract of *L. casei* subs. *rhamnosus* (*L.GG*) and *L. casei* MB451. As shown in Figures 2 and 3, the cell debris of *lactobacilli* only had a weak stimulating capacity at concentrations of less than 10^7 CFU/mL.

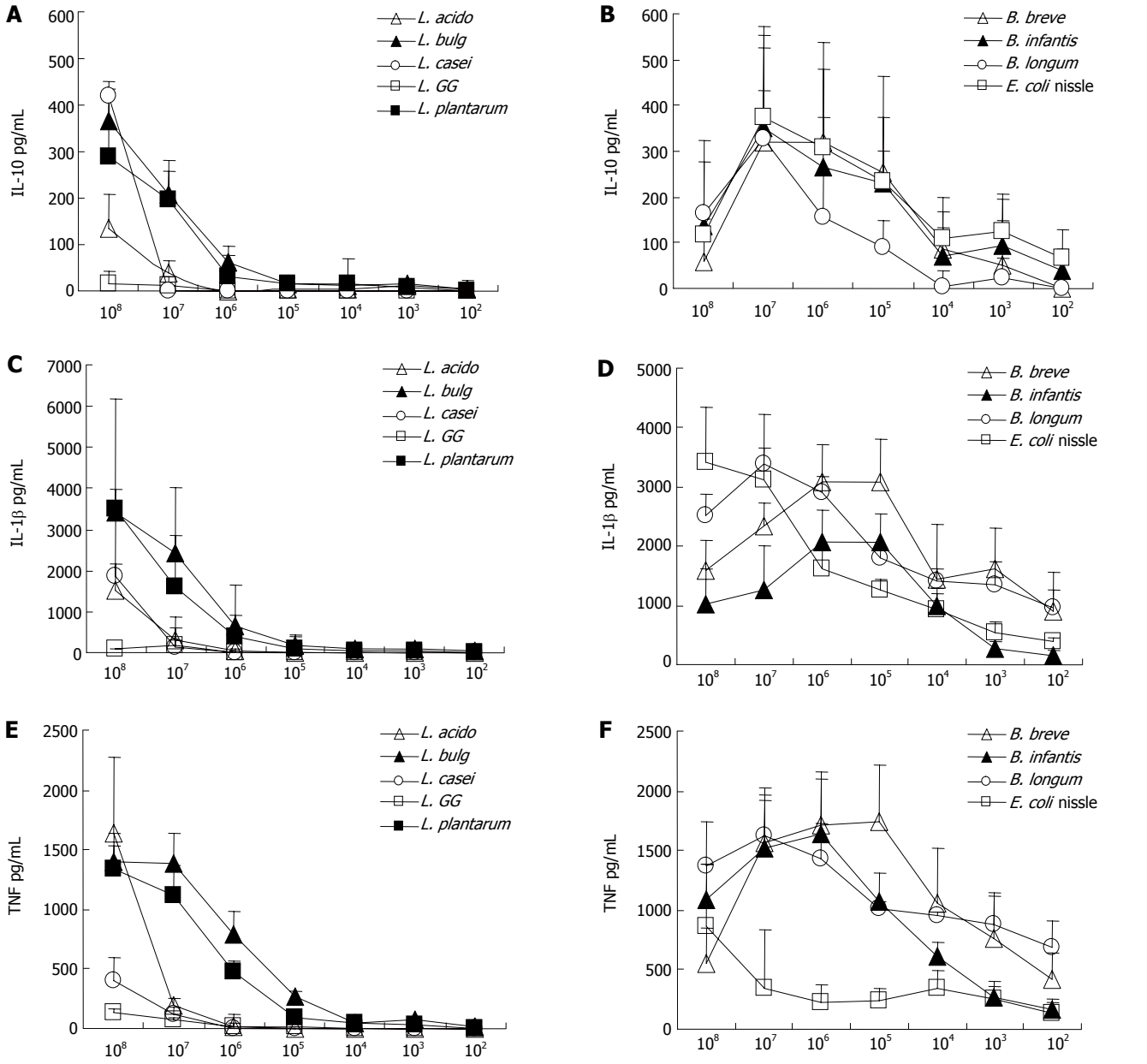

Figure 2 Cytokine concentration of supernatant after incubation of PBMNC with cell debris of bacteria in different concentrations. **A** and **B**: supernatant concentration of IL-10 in pg/ml (mean ± SE); **C** and **D**: supernatant concentration of IL1β in pg/ml (mean ± SE); **E** and **F**: supernatant concentration of TNFα in pg/mL (mean ± SE). *Lactobacilli* are described on the left side and *bifidobacteria* are described on the right side.

***Bifidobacteria*:** Cell debris of each *bifidobacteria* strain led to higher TNFα concentrations in supernatant of PBMNC than their cell extract (AUC: *B. breve* Y8 cell debris: 7645.5 ± 1823.1 pg/mL; cell extract: 180.1 ± 85.5 pg/mL; AUC *B. longum* Y10 cell debris: 6951.2 ± 522.7 pg/mL; cell extract: 83.6 ± 40.0 pg/mL; AUC *B. infantis* Y1 cell debris: 5584.8 ± 1098.8 pg/mL; cell extract: 290.5 ± 103.6 pg/mL) ($P < 0.05$). No difference was found between *bifidobacteria* species when the stimulating capacity of cell extract or cell debris was compared. The cell debris from *bifidobacteria* led to higher TNFα concentrations in supernatant of PBMNC than cell debris from *lactobacilli* or *E. coli* nissle (AUC cell debris of *bifidobacteria*: 6882.4 L ± 1355.0 pg/mL; AUC cell debris of *lactobacilli*: 1630.8 ± 1265.1 pg/mL; AUC cell debris of *E. coli* nissle: 1466.6 ± 1356.0 pg/mL) ($P < 0.02$) (Figure 4). As shown in Figure 2, the cell debris from *B. breve* Y8 and *B. longum* Y10 even had a stimulating capacity at low concentrations (10^3 CFU/mL and 10^2 CFU/mL), whereas the TNF-α production in PBMNC was weak when incubated with *B. infantis* Y1 at a concentration of less than 10^4 CFU/mL. The highest concentration (10^8 CFU/mL) of *bifidobacteria* had no strong stimulating ability to produce TNF-α in PBMNC, whereas the viability measured by trypan blue test was over 97%.

***E. coli* nissle:** High concentrations (10^8 CFU/mL) of cell debris of *E. coli* nissle led to high concentrations of TNF-α, whereas lower concentrations of cell debris and extracts led to lower concentrations of TNF-α (Figure 3) (AUC cell debris: 1466.6 ± 1356 pg/mL; cell extract: 1153.0 ± 748.4 pg/mL) The cell debris of *E. coli* nissle

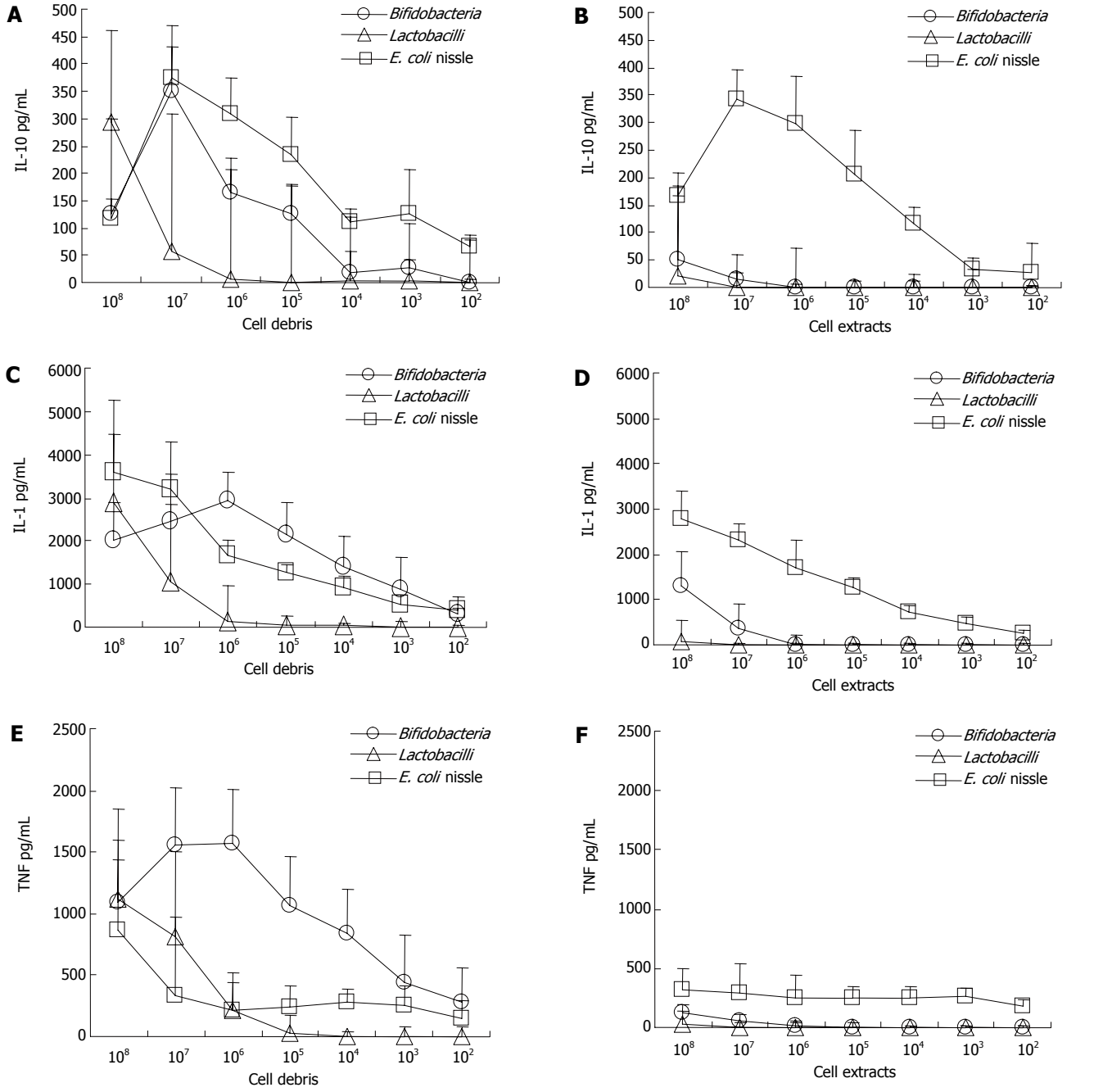

Figure 3 Cytokine concentrations of supernatant after incubation of PBMNC with cell debris (left side) or cell extracts (right side) of bacteria from different species families in different concentrations. **A** and **B**: supernatant concentration of IL-10 in pg/ml (mean ± SE); **C** and **D**: supernatant concentration of IL1β in pg/ml (mean ± SE); **E** and **F**: supernatant concentration of TNFα in pg/mL (mean ± SE).

had a similar stimulating capacity similar to *lactobacilli* but a significantly less capacity than that of *bifidobacteria* ($P < 0.02$) (Figure 4).

Inhibition of stimulated PBMNC by bacterial cell debris and extract

None of the bacterial cell debris and extract of *lactobacilli, bifidobacteria* or *E. coli* nissle had an inhibitory effect on the cytokine production in PBMNC. Pre-incubation with cell debris or with cell extract and stimulation with LPS led to similar cytokine production of LPS alone or cell debris alone (data not shown).

DISCUSSION

Different probiotic strains used in clinical trials have shown prophylactic properties in different inflammatory diseases of the gastrointestinal tract, such as Crohn's disease, ulcerative colitis, pouchitis, antibiotic-associated colitis and traveller's diarrhoea[27-28]. Recently, we have shown that the IL-10 concentration in the mucosa of ileo- anal pouch tissue is elevated after administration of probiotics[25]. In this *in vivo* approach we used a highly concentrated probiotic preparation containing different lactic acid bacteria. Our hypothesis is that a high concentration of bacteria contributes to these clinical results and

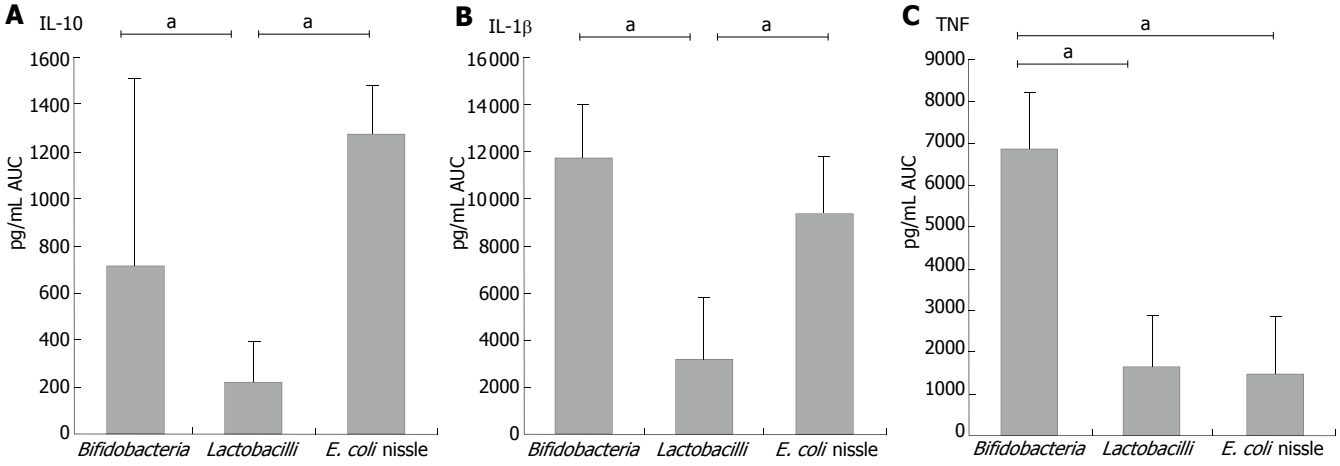

Figure 4 Supernatant concentrations of different cytokines after co-incubation of PBMNC with cell debris of bacteria from different species families. With regard to *lactobacilli* results are pooled from *L.acidophilus*, *L.bulgaricus*, *L.casei*, *L.GG*, *L.plantarum*. With regard to *bifidobacteria* results are pooled from *B.breve*, *B.infantis*, *B.longum*. IL-10, IL-1β and TNFα shown as area under the curve (mean ± SE) ($^{a}P < 0.05$).

immunologic findings[3]. However, the specific property of different strains remains unclear. Therefore, we investigated an *in vitro* model in order to test different probiotic strains and species, which are used in clinical practice to prevent inflammatory diseases, in order to understand the pro- and anti-inflammatory properties of specific strains. There are several studies on the induction of cytokines by cell components of lactic acid bacteria to induce cytokines[29-31]. However, systematic analysis of probiotic bacteria used in clinical practice for the prevention of inflammatory disease, has not been performed until now in a human cell model. For this purpose we used PBMNC which are easily available and express toll-like-receptor (TLR) 2 and TLR 4 as well as CD14 which are shown to mediate immune response to microbial components as peptidoglycan and lipoteichoic acid[32-33]. Until now there is no report on comparison of dose response over a broad range of different concentrations of probiotic bacteria used in clinical practice for prevention of inflammatory bowel disease. Our findings on PBMNC indicate that stimulation by *lactobacilli* works in a dose-dependent way. High doses of cell debris could stimulate PBMNC to produce pro- and anti-inflammatory cytokines. The cell extract has a less stimulating capacity in a dose-dependent manner. An interesting finding is that the cell debris of *L. delbrueckii* subsp. *bulgaricus* MB453 and *L. plantarum* MB452 stimulates PBMNC when used at concentrations higher than 10^4 CFU/mL, while both *L. azidophilus* MB443 and *L. casei* MB451 strains only require concentrations higher than 10^6 and 10^5 CFU/mL. Cell debris of *L. casei* supsp. *rhamnosus* (*L.GG*) had a very low stimulating capacity compared to other strains (Figure 1). The weak or even absent reaction at high concentrations of *lactobacilli* (10^6 or 10^5 CFU/mL) is not suspected. This phenomenon is reproducible in different blood donors and exists in all different *lactobacilli* strains when used for this examination. Schultz and co-workers[34] recently showed that *L. casei* subsp. *rhamnosus (L.GG)*, which had the lowest stimulating capacity in our study, induces immunologic tolerance to granulocytes after oral administration for several weeks. Since *lactobacillus* strains normally are early inhabitants of the human gastrointestinal tract, oral tolerance to low concentrations of *lactobacillus* strains might generally develop[35]. Although this is expected for *bifidobacteria*, they stimulate pro- and anti-inflammatory cytokines more significantly than *lactobacilli*. But the stimulation pattern is different. The highest concentration of *bifidobacteria* induces PBMNC to produce less pro- and anti-inflammatory cytokines than lower concentrations of the strain. Whether the lower induction of cytokine release in incubation with highly concentrated cell debris is due to deletion or apoptosis of PBMNC remains unclear. Toxic reaction or a reaction resulting in direct cell death can be excluded by the trypan blue control test which provides information about the functionality of cell membrane but not about the metabolic condition of cells. Recently, it has been proposed in a different model that *bifidobacteria* strains induce oral tolerance[36] but also induction of oral tolerance to *E. coli* and *lactobacilli* has been reported[37-38]. The stimulating capacity of *E. coli* nissle shows a different pattern. The cell extract and debris of *E. coli* nissle have a similar ability to produce cytokines. Interestingly, the cell debris and extract of *E. coli* nissle at low concentrations can stimulate epithelial HT29/19 cells to produce the chemotactic factor interleukin-8 (IL-8), whereas the cell debris and extract of *lactobacilli* and *bifidobacteria* do not stimulate epithelial HT29/19 cells to release IL-8[38]. *E. coli* nissle, which has been shown to be effective in maintaining remission of ulcerative colitis, has a high stimulating capacity for IL-10 and IL-1β compared to other strains, but a low capacity for TNF-α. *Bifidobacteria* of the probiotic composition VSL#3 of b*ifidobacteria*, which can prevent inflammatory bowel disease, can stimulate PBMNC to produce IL-10[9,10]. *L. GG* can weakly stimulate PBMNC to produce IL-10 and has no positive effect on inflammatory bowel disease[7-8]. This is consistent with the findings in another study[25]. *L. GG* has been primarily used in trials for prevention of relapses in Crohn's disease. It has been recently reported that Crohn's disease is associated with the polymorphism of the nucleotide-binding oligomerization domain 2

(NOD 2)[39-40]. NOD 2 is a regulator of TLR 2-mediated response to microbial agents[41] and Gram-positive bacteria like *lactobacilli* are typical ligands for TLR 2[32]. Since the function of mutations in the NOD 2 gene in Crohn's disease is not clear[42], explanation about the lacking effect of probiotics on Crohn's disease is warranted.

In conclusion, the ability of probiotic bacteria to stimulate PBMNC is different. Compared to *E. coli* nissle and *bifidobacteria*, *lactobacilli* debris exerts effects only at high concentrations. Whereas the extract of *lactobacilli* and *bifidobacteria* has only weak effects, while the cell extract and debris of *E. coli* nissle have similar effects. The higher IL-10 response to *E. coli* nissle and *bifidobacteria* corresponds to the positive effect of these probiotic strains on inflammatory bowel disease compared to negative outcomes obtained with *lactobacilli*.

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BASIC RESEARCH

Effects of probiotic bacteria on gastrointestinal motility in guinea-pig isolated tissue

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Abstract

AIM: To evaluate the intestinal motility changes evoked by 8 bacterial strains belonging to *Bifidobacterium*, *Lactobacillus* and *Streptococcus* genera within the probiotic preparation VSL#3.

METHODS: Ileum and proximal colon segments isolated from guinea-pigs were used as a study model. Entire cells and cell fractions (cell debris, cell wall fraction, cytoplasmatic fraction, proteinaceous and non-proteinaceous cytoplasmatic components) of VSL#3 strains and, as controls, *Escherichia coli*, *Salmonella aboni* and *Bacillus licheniformis* were tested in this *in vitro* model.

RESULTS: Among the bacterial cell fractions tested, only the cytoplasmatic fraction modified intestinal motility. *Lactobacillus* strains stimulated the contraction of ileum segment, whereas all probiotic strains tested induced proximal colon relaxation response. The non-proteinaceous cytoplasmatic components were responsible for the colon relaxation.

CONCLUSION: The results obtained in this study suggest that the proximal colon relaxation activity showed by the probiotic bacteria could be one of the possible mechanisms of action by which probiotics exert their positive effects in regulating intestinal motility.

Key words: Probiotics; Intestinal motility; Guinea-pigs; Ileum; Colon

INTRODUCTION

Probiotics are viable microbial cells that upon ingestion in specific numbers appear to have beneficial effects on the health and well-being of the host, beyond inherent basic nutrition[1]. These health-promoting effects are predominantly related to reinforcement of the intestinal mucosal barrier against enteropathogens' colonization, immunostimulation and immunomodulation, anticarcinogenic and antimutagenic activities, improvement of lactose utilization, and reduction of serum cholesterol[2].

Most probiotics are bacteria members of the genera *Lactobacillus* and *Bifidobacterium*, which represent important components of human gastrointestinal flora. However, other nonpathogenic bacteria, such as *Streptococcus*, some strains of *Escherichia coli* and *Enterococcus faecium*, and yeasts, such as *Saccharomyces boulardii*, have been used in probiotic preparations[3].

Experimental and clinical studies support the use of probiotics in the treatment of intestinal disorders such as infectious diarrhea[4-7], antibiotic diarrhea[8-10], traveller's diarrhea[11-13], irritable bowel syndrome or functional diarrhea[14-18] and inflammatory bowel disease[19-23]. In recent double-blind placebo controlled trials the efficacy of the probiotic preparation VSL#3 has been shown as maintenance treatment and prophylactic therapy in patients with diarrhea-predominant irritable bowel syndrome and pouchitis[18,22-24]. VSL#3 (VSL Pharmaceuticals, Ft. Lauderdale, FL, USA) contains a mixture of eight different bacterial species at very high concentrations (450 billions/sachet of viable lyophilized bacteria). The preparation consists of three strains of *bifidobacteria* (*Bifidobacterium longum*, *Bifidobacterium breve*, *Bifidobacterium infantis*), four strains of *lactobacilli* (*Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus plantarum*) and one strain of *Streptococcus thermophilus*.

To the best of our knowledge, there are no experimental data evaluating the effects of probiotic bacteria on

the intestinal motility. Functional assays could represent useful tools to investigate the effects evoked by the entire probiotic cells and/or their fraction on different segments of the intestinal tissue.

The aim of this study was to evaluate the effect of the eight strains within the probiotic preparation VSL#3[18,21-23] on intestinal motility using ileum and proximal colon segments isolated from guinea-pigs. Entire bacterial cells, cell fractions of VSL#3 strains, and, as controls, *Escherichia coli*, *Salmonella aboni* and *Bacillus licheniformis*, were tested in this *in vitro* model.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The following bacterial strains were used in this study: *Bifidobacterium breve*, *Bifidobacterium infantis*, *Bifidobacterium longum*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus plantarum* and *Streptococcus thermophilus*.

Bifidobacterium and *Lactobacillus* strains were grown anaerobically (Anaerobic System, Model 2028, Forma Scientific, Marietta, OH, USA) in MRS medium (Difco, Detroit, MI, USA) supplemented with 0.05% L-cysteine hydrochloride monohydrate (Merck, Darmstadt, Germany), at 37℃. *S. thermophilus* was cultured anaerobically in M17 medium (Difco) at 37℃. *Salmonella aboni* NCTC 6017, *Escherichia coli* ATCC 11105 and *Bacillus licheniformis* BGSC 5A24 were used as controls and grown aerobically in LB medium (Difco) at 37℃.

Preparation of bacterial cell fractions

Ten milliliter of the bacterial mid log cultures, counted by plating technique on the previously mentioned media, was collected by centrifugation (5000 × *g* at 4℃ for 5 min), washed, and resuspended in 1 mL of De Jalon buffer (155 mmol/L NaCl, 5.6 mmol/L KCl, 0.5 mmol/L $CaCl_2$, 6.0 mmol/L $NaHCO_3$, 2.8 mmol/L glucose)[25], obtaining a final bacterial concentration of 1×10^9-1×10^{10} colony forming units (CFUs/mL). Subsequently the bacterial suspensions were sonicated (Bronson Sonifier W-250, Heineman, Schwäbisch, Germany) at power level 2 at 20% duty for 8 min and centrifuged at 8000 × *g* for 30 min to separate cell debris from crude extract. Cell debris was resuspended in 1 mL of De Jalon buffer. The crude extract was ultracentrifuged at 250 000 × *g* at 4℃ for 2 h: the supernatant represents the cytoplasmatic fraction while the pellet, resuspended in 1 mL of 50 mmol/L TRIS.HCl pH 7.6, represents the fraction enriched in membrane proteins.

In order to obtain the bacterial cell wall fraction, bacterial cultures were centrifuged at 5000 × *g* at 4℃ for 5 min, washed in 50 mmol/L Tris HCl pH 7.6, resuspended in 1 mL of protoplast buffer [50 mmol/L Tris HCl pH 7.6, 1 mol/L sucrose, 50 mL of complete™ protease inhibitor (Roche, Mannheim, Germany), 15 mg/mL lysozyme] and incubated at 37℃ for 20 min. The supernatant was recovered by centrifuging at 5000 × *g* at 4℃ for 5 min.

Proteinaceous components were precipitated by addition of 9 volumes of acetone:HCl (10:0.1) to one volume of the cytoplasmatic fraction and collected by centrifuging at 12 000 × *g* at 4℃ for 5 min. Supernatant (non-proteinaceous fraction) and protein pellet were dried on ice under vacuum for removing acetone and resuspended in the initial volume of 50 mmol/L TRIS. HCl pH 7.6. The very low value of pH (about 1) of the non-proteinaceous fraction was adjusted to a value of 7.4 to overcome the unspecific colonic contractile response due to the acid pH. Similarly, the cytoplasmatic fraction was incubated with proteinase K (500 mg/mL) at 50℃ for 1 h for the enzymatic digestion of the proteins. All the bacterial fractions of cell debris (i.e., cell wall fraction, membrane proteins, cytoplasmatic fractions, proteinaceous and non-proteinaceous cytoplasmatic components) were aliquoted and stored at -80℃ before to be used in the *in vitro* stimulation of ileum and proximal colon.

SDS PAGE

Ten μL of *B. infantis* cytoplasmatic fraction, non-proteinaceous fraction and cytoplasmatic fraction deprived of proteins by proteinase K digestion was analyzed by SDS-PAGE, as described by Laemmli[26], using a 12% polyacrylamide running gel. The gels were stained with silver nitrate.

Bacterial DNA preparation

Isolation of genomic DNA from pure cultures of the probiotic bacteria was performed as previously described[27]. In order to obtain the complete cell disruption, the method was slightly modified by prolonging the enzymatic lysis for 1 to 3 h and grinding with glass beads (150-212 μm, Sigma, St. Louis, MO, USA). Concentration and purity of all DNA preparations were determined by measuring OD_{260} absorbance and $OD_{260/280}$ ratio, respectively. Only DNAs with an $OD_{260/280}$ ratio > 1.8 were used.

Animals and preparation of Guinea-pig ileum and proximal colon

Guinea-pigs of either sex (200-400 g) obtained from Charles River (Calco, Como, Italy) were used. The animals were housed according to the ECC Council Directive regarding the protection of animals used for experimental and other scientific purposes. All procedures followed the guidelines of The Animal Care and Use Committee of The University of Bologna (Bologna, Italy). The animals were sacrificed by cervical dislocation, and the organ (ileum or proximal colon) required was set up rapidly under a suitable resting tension in 15-mL organ baths containing appropriate physiological salt solution (PSS) consistently warmed (see below) and buffered to pH 7.4 by saturation with 950 mL/L O_2 and 50 mL/L CO_2 gas.

Preparation of ileum

The terminal portion of ileum (3-4 cm near the ileo-caecal junction) was cleaned with Tyrode solution of the following composition: 118 mmol/L NaCl, 4.75 mmol/L KCl, 2.54 mmol/L $CaCl_2$, 1.20 mmol/L $MgSO_4$, 1.19 mmol/L $KH_2PO_4 \cdot 2H_2O$, 25 mmol/L $NaHCO_3$, and 11 mmol/L glucose. The mesenteric tissue was removed. The ileum tissue was cut in two segments of 2-3 cm taken in the lon-

gitudinal direction along the intestinal wall. The segments were set up upright under 1-g tension in a jacketed tissue bath (15 mL, 37℃) containing Tyrode solution buffered to pH 7.4 by saturation with 950 mL/L O_2 and 50 mL/L CO_2 gas. Tissues were allowed to equilibrate for at least 30 min, during which time the bathing solution was changed every 10 min. After stabilization, the strips were challenged with 1 μmol/L carbachol (Sigma, Italy) to assess the responsive capacity of the tissue[28].

Preparation of proximal colon

Starting approximately 1 cm distal from the caecocolonic junction, a segment of about 3 cm was excised, cleansed by rinsing it with De Jalon solution[25], and the mesenteric tissue was removed. The proximal colon segment was cut in two segments of about 1 cm each taken in the longitudinal direction along the intestinal wall. The segments were set up upright under 1-g tension at 37℃ in a jacketed tissue bath (15 mL, 37℃) containing De Jalon solution buffered to pH 7.4 by saturation with 950 mL/L O_2 and 50 mL/L CO_2 gas. Tissues were allowed to equilibrate for at least 30 min during which time the bathing solution was changed every 10 min. After stabilization, the strips were challenged with 5 μmol/L 5-hydroxytryptamine (5-HT) (Sigma, Italy) in the presence of 1 μmol/L atropine (Sigma, Italy) to assess the responsive capacity of the tissue.

Functional assays on ileum and proximal colon

After stabilization and assessment of the responsive capacity of the tissue in the organ bath, concentration-response curves were constructed by cumulative addition of the above described bacterial preparations (1 to 1500 μL). Each successive addition of bacterial preparations was performed after the response to the previous addition reached its maximum level and remained steady. Longitudinal muscle contractions or relaxations were recorded isometrically by securing one end of the tissue segments to a tissue holder and the other end to a force displacement transducer (FT. 03, Grass Instruments, Quincy, MA) using Power Lab software (ADInstruments Pty Ltd, Castle Hill, Australia). Each tracing was obtained by using separate intestinal strips.

Statistical analysis

Experiments were performed in duplicate with tissue from the same animal, and mean values were recorded. All data are presented as mean ± SE (n = 3-5 for ileum and n = 5-7 for proximal colon). Differences between means were calculated with Student t-test and P-values < 0.05 were considered statistically significant[29-31].

RESULTS

Bacterial effect on motility response in guinea-pig ileum

The motility response of the guinea-pig ileum segment was investigated with increasing concentrations of the following components of the probiotic VSL#3 mixture: live bacteria, bacterial cell debris, and crude extracts. VSL#3 live bacteria and cell debris did not modify the guinea-pig ileum motility (data not shown), whereas crude extracts dose-dependently increased both spontaneous phasic and tonic contractions of the ileum (Figure 1A). The effects on motility induced by crude extracts persisted even after adjusting the pH of the solution from around 5.0, as found in the crude extract, to pH 7.4 and were reversible as the basal tone returned to initial values after

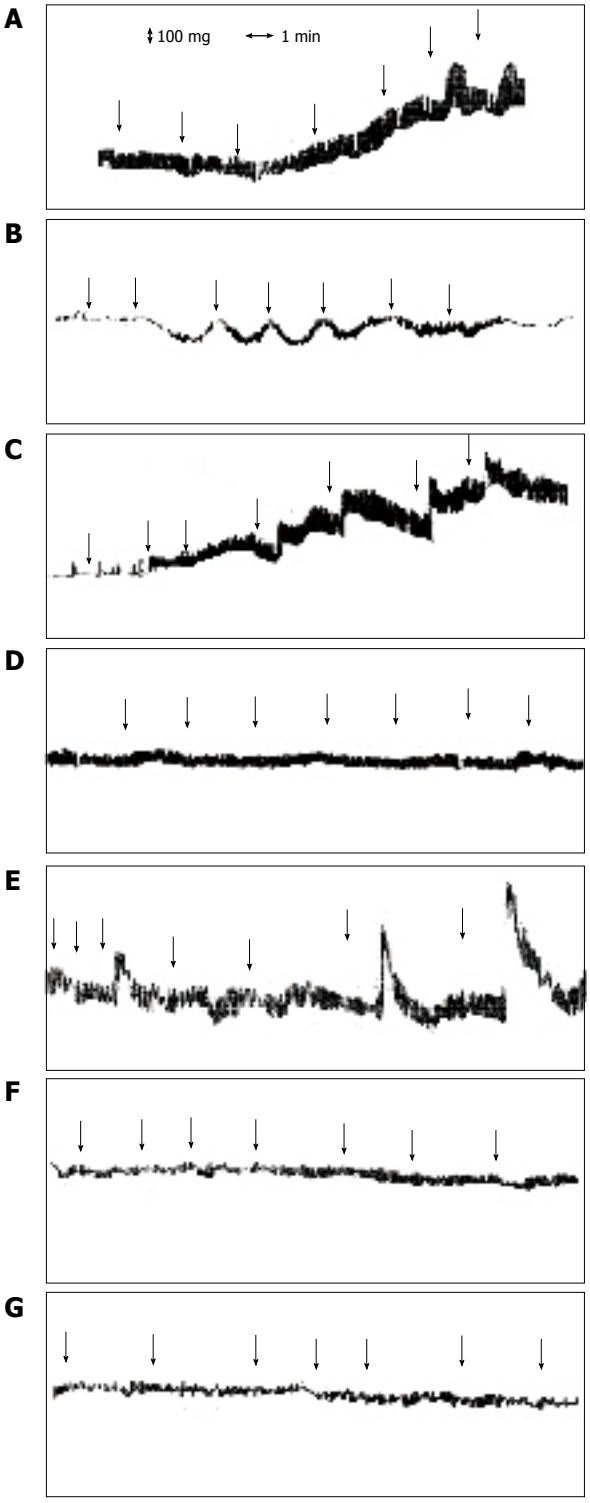


Figure 1 Recorder tracing of the cumulative dose of VSL#3 (**A**), *Bifidobacterium* (**B**), *Lactobacillus* (**C**), *Streptococcus thermophilus* (**D**), *Salmonella aboni* (**E**), *Escherichia coli* (**F**), and *Bacillus licheniformis* (**G**) crude extracts on the contractility of Guinea-pig isolated ileum. Arrows indicate the addition of 50, 100, 150, 200, 250, 300 and 350 μL of each crude extract tested.

several washing steps (data not shown).

To identify the microbial genera within the VSL#3 cocktail responsible for the effects on motility in the guinea-pig ileum, crude extract mixtures of *Bifidobacterium* (*B. longum*, *B. infantis*, *B. breve*), *Lactobacillus* (*L. acidophilus*, *L. casei*, *L. plantarum*, *L. bulgaricus*), and *Streptococcus thermophilus* were individually tested. The following differences in the motility response by the crude extracts of the three probiotic genera were observed: (1) *Lactobacillus* strains induced a concentration-dependent contraction of the ileum, which was rapid in onset; (2) *Bifidobacterium* promoted a weak and transient stimulation of the ileum in a non concentration-dependent manner; (3) *Streptococcus* did not promote any significant response (Figures 1B-D).

The intestinal bacteria *Salmonella aboni* and *Escherichia coli* and the non-intestinal bacterium *Bacillus licheniformis* were used as controls to verify the specificity of the probiotic effects on gut motility (Figures 1E-G). Ileum motility was not affected by the exposure to *E. coli* and *B. licheniformis* crude extracts (Figures 1F, G), whereas *S. aboni* crude extracts were able to trigger the ileum contraction in a dose-dependent manner (Figure 1E). Furthermore, the amplitude of contraction induced by *S. aboni* was significantly higher than that promoted by the VSL#3 mixture and *Lactobacillus* crude extracts.

In order to verify the involvement of muscarinic receptors in the observed contraction response, the muscarinic antagonist atropine (1 μmol/L) was added to the organ bath. Atropine did not modify the motility response of ileal tissue exposed to all the bacterial crude extracts tested (data not shown).

Bacterial effect on motility response in guinea-pig proximal colon

The motility response of guinea-pig proximal colon segment was investigated using a similar protocol as with the guinea-pig ileum by adding identical concentrations of the bacterial cell preparations tested in the ileum stimulation study. Similar to the guinea-pig ileum, only the crude extracts of the probiotic VSL#3 mixture showed a significant effect on colon motility. However, the effect on the guinea-pig proximal colon was opposite to that observed in ileum (Figure 2A). In particular, the crude extract of the VSL#3 mixture elicited a rapid and sustained relaxation of the colon tissue, characterized by peaks in response to each addition, together with a progressive lowering of the basal tone. As previously carried out with the ileum, crude extract preparations at pH 5 and 7.4 were tested obtaining the same colon relaxation response (data not shown).

The VSL#3 extract mixtures of the genera *Bifidobacterium*, *Lactobacillus*, and *S. thermophilus* were assessed (Figures 2B-D). All the bacterial groups provoked a dose-dependent relaxation of the colon tissue, but no significant motility effect was observed by exposure to the control bacteria (Figures 2E-G). Clear colon relaxation effects were promoted by the crude extracts of *Bifidobacterium* strains and *S. thermophilus* and were quite similar to those induced by the crude extracts of the VSL#3 mixture (Figure 3). *Lactobacillus* strain's crude extract showed a lower relaxation response. Washings of the colon segment exposed to

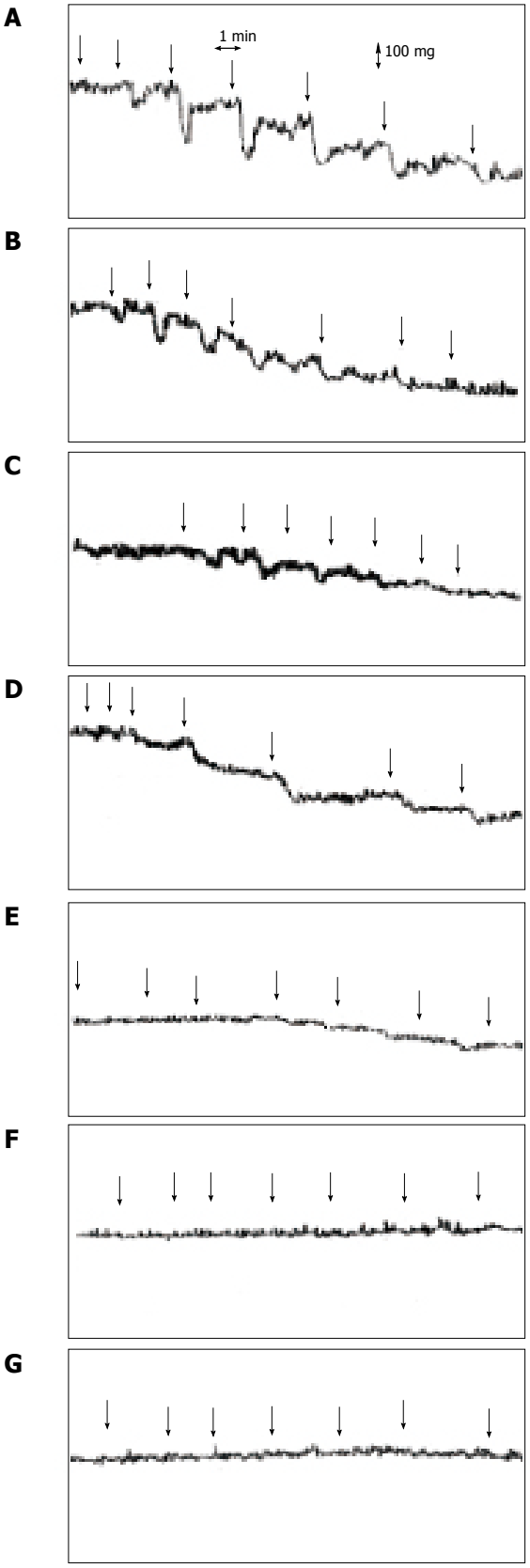
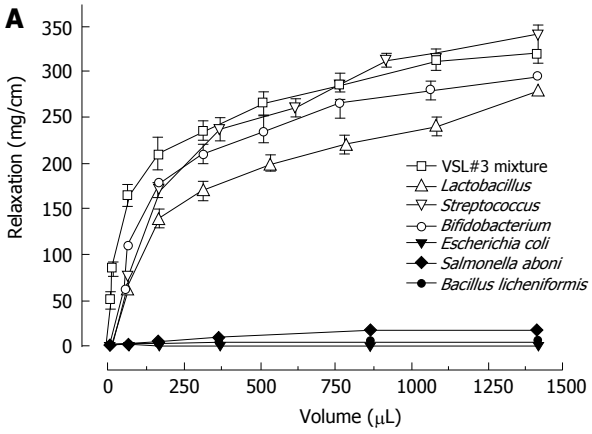


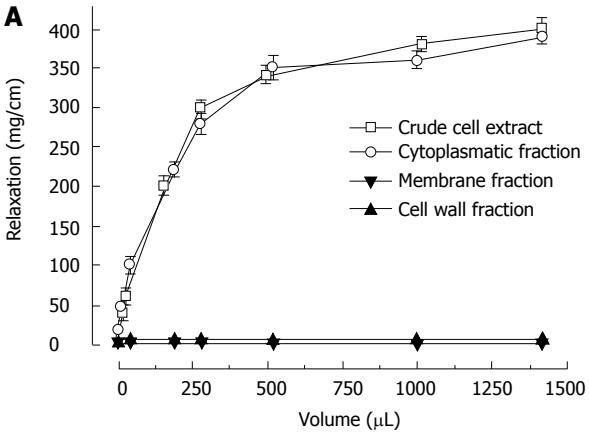
Figure 2 Recorder tracing of the cumulative dose of VSL#3 (**A**), *Bifidobacterium* (**B**), *Lactobacillus* (**C**), *Streptococcus thermophilus* (**D**), *Salmonella aboni* (**E**), *Escherichia coli* (**F**), and *Bacillus licheniformis* (**G**) crude extracts on the contractility of Guinea-pig isolated proximal colon. Arrows indicate the addition of 50, 100, 150, 200, 250, 300 and 350 μL of each crude extract tested.



B

Bacterial strains	μL	Relacation %
VSL#3 mixture	1100	100
Lactobacillus	1400	86 ± 3.9
Streptococcus	1400	106 ± 7.5
Bifidobacterium	1050	92 ± 1.3
Escherichia coli	1400	0
Salmonella aboni	1400	0
Bacillus licheniformis	1400	6 ± 0.4

Figure 3 Relaxation induced by crude extract of VSL#3 mixture, *Lactobacillus*, *Streptococcus*, *Bifidobacterium*, *Escherichia coli*, *Salmonella aboni* and *Bacillus licheniformis* preparations on Guinea-pig proximal colon. Relaxation was expressed as change in mg-tension per cm of chart paper in resting tone (**A**). Relaxation was expressed in percentage, by considering VSL#3 mixture-induced maximal relaxation as 100% (**B**). The volume (μL) indicates the minimal value exerting maximal relaxation effects. Each crude extract was prepared from a bacterial suspension equivalent to a concentration of 3.5 x 10^9 CFUs/mL. Each point is the mean ± SE of 5 to 7 observations. Mean ± SE was given ($P < 0.05$).



B

B. infantis	μL	Relacation %
Crude cell extract	1400	100
Cytoplasmatic fraction	1400	96 ± 3.5
Membrane fraction	1400	0
Cell wall fraction	1400	0

Figure 4 Relaxation induced by *B. infantis* crude extract, cytoplasmatic fraction, membrane fraction and cell wall fraction on Guinea-pig proximal colon. Relaxation was expressed as change in mg-tension per cm of chart paper in resting tone (**A**). Relaxation was expressed in percentage, by considering crude extract-induced maximal relaxation as 100% (**B**). The volume (μL) indicates the minimal value exerting maximal relaxation effects. Each crude extract was prepared from a bacterial suspension equivalent to a concentration of 3.5 x 10^9 CFUs/mL. Each point is the mean ± SE of 5 to 7 observations. Mean ± SE was given ($P < 0.05$).

successive additions of all crude extracts tested restored the basal tone of the tissue (data not shown). A further investigation was carried out with the crude extracts of VSL#3 *B. infantis* and *L. casei* strains, representative of *Bifidobacterium* and *Lactobacillus* genera. Colon relaxation patterns induced by these samples were identical to those obtained with the crude extracts of *Bifidobacterium* and *Lactobacillus* mixtures (data not shown).

Effects of B. infantis cell components on colon motility

To confirm that bacterial cytoplasmatic components are mainly responsible for colon relaxation, a more refined fractioning of the *B. infantis* cells was performed by separating cell wall fraction, membrane proteins, and cytoplasmatic fraction. A concentration-response curve was constructed for each of these bacterial portions (Figure 4). Indeed, the cytoplasmatic fraction showed a sustained relaxation of the colon segment equal to that demonstrated by the *B. infantis* crude extract. As expected, the cell wall fraction and the membrane proteins did not produce any significant effect.

The cytoplasmatic fraction of *B. infantis* was further refined in proteinaceous and non-proteinaceous components. The absence of protein in the non-proteinaceous fraction was verified by SDS PAGE analysis (Figure 5). As reported in Figure 6, the effect on colon relaxation was induced by the non-proteinaceous components, which showed a motility response similar to

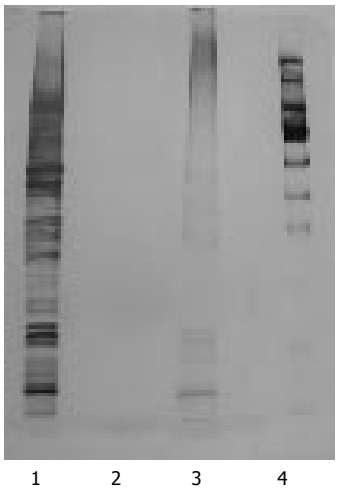
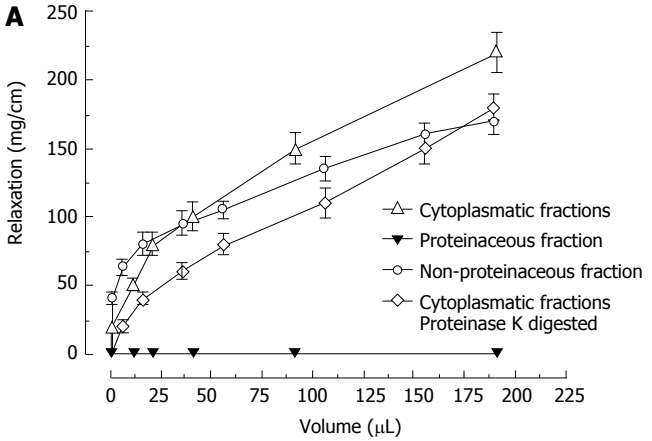


Figure 5 SDS PAGE electrophoresis of *B. infantis* cytoplasmatic fraction (lane 1), cytoplasmatic non-proteinaceous components (lane 2), cytoplasmatic fraction proteinase K digested (lane 3), molecular weight marker (lane 4).

that exerted by the cytoplasmatic fraction. The relaxation effect exerted by the non-proteinaceous components was confirmed by colon motility experiments carried out with a *B. infantis* cytoplasmatic fraction deprived of proteins by proteinase K digestion. This preparation, in which the enzymatic protein degradation was demonstrated by SDS PAGE analysis (Figure 5), evoked a significant colonic relaxation response similar to that obtained with the buffered non-proteinaceous preparation (Figure 6). To further characterize the *B. infantis* non-proteinaceous cytoplasmatic components, the possible involvement of bacterial genomic DNA fraction was tested. Addition to the organ bath of *B. infantis* DNA ranging from 0.5 μg to 10 μg did not modify the colon motility (data not shown).

A



B

B. infantis	μL	Relacation %
Cytoplasmatic fractions	190	100
Proteinaceous fraction	190	0
Non-proteinaceous fraction	190	77 ± 3.7
Cytoplasmatic fractions proteinase K digested	190	81 ± 2.4

Figure 6 Relaxation induced by *B. infantis* cytoplasmatic fraction, cytoplasmatic proteinaceous components, cytoplasmatic non-proteinaceous components and cytoplasmatic fraction proteinase K digested on guinea-pig proximal colon Relaxation was expressed as change in mg-tension per cm of chart paper in resting tone (**A**). Relaxation was expressed in percentage, by considering cytoplasmatic fraction-induced maximal relaxation as 100% (**B**). The volume (μL) indicates the minimal value exerting maximal relaxation effects. Each crude extract was prepared from a bacterial suspension equivalent to a concentration of 3.5 x 10^9 CFUs/mL. Each point is the mean ± SE of 5 to 7 observations.

DISCUSSION

The gut represents a complex and dynamic microbial ecosystem in which intestinal microflora has important and specific metabolic, trophic, and protective functions. Normal gut structure and function are the end-point of a complex set of interactions between the host and microorganisms colonizing the gut[2]. Several studies have shown that probiotics restore mucosal integrity and regulate the immune response. However, the effects of probiotics on the enteric nervous system and the intestinal musculature have not been systematically studied. This study was designed to investigate the motility response evoked by the probiotic preparation VSL#3 on the ileum and proximal colon isolated from guinea-pigs.

The choice of VSL#3, a formulation with a high viable concentration of *Bifidobacterium*, *Lactobacillus* and *Streptococcus thermophilus*, was based on the reported efficacy in bowel diseases associated with changes in propulsive motility of the gut, including diarrhea-predominant irritable bowel syndrome[18]. Furthermore, in clinical trials these exogenous probiotic bacteria have been shown to transiently colonize the human gut[19, 21, 22].

The data from this study show that the motility of isolated ileum is not influenced by the addition of either entire cells or cell debris of the VSL#3 mixture, whereas its crude extracts induce a dose-dependent contraction. It is noteworthy that VSL#3 crude extracts, which have a pH of about 5, and crude extracts in which the pH was increased up to physiologic value of 7.4 showed similar contractile responses. These results demonstrate that the ileum motility response is not related to the pH of the bacterial fraction and suggest that some bacterial metabolites could be involved in the contractions. Furthermore, as the presence of atropine (1 μmol/L) in the organ bath throughout the experiments did not determine a decrease of contraction induced by the addition of the VSL#3 crude extracts, the mechanism of action of the bacterial fractions does not involve muscarinic receptors.

Crude extracts of the different bacterial groups within the VSL#3 preparation provoke different motility response patterns. *Bifidobacterium* and *Streptococcus* strains did not change the basal tone of the ileum tissue, while *Lactobacillus* strains induced relevant ileum contractions. However, this contractile response is observed with volumes of cell extracts corresponding to *lactobacilli* concentration values of at least 10^7 CFU/mL, a value that is generally not detectable at ileum level of healthy humans[27]. This result suggests that the contractile effect evoked in ileum by VSL#3 cell extracts will be of minor importance *in vivo* at ileum level, as the rapid transit of ileum content and the low bacterial titer do not allow it to reach sufficient bacterial lysis or metabolic secretion. Furthermore, it is noteworthy that the contractile response induced by *Lactobacillus* is lower than that promoted by crude extracts of intestinal *S. aboni* added at the same concentration.

Like the guinea-pig ileum, the motility of guinea-pig proximal colon segment is modified by crude extracts of the VSL#3 mixture, while entire bacterial cells and cell debris do not exert any effect. The crude extract of all the probiotic strains (*Bifidobacterium, Lactobacillus* and *S. thermophilus*) included in VSL#3 induced dose-dependent relaxation of the proximal colon tissue, whereas the crude extracts of the control strains did not influence colon motility. These results suggest that the ability to induce a colon relaxation response is specific for these probiotic bacteria. Furthermore, the proximal colon relaxation response induced by probiotic bacteria cell extracts is reversible as successive washings abolished the colon relaxation. The restoration of the basal tone of the proximal colon tissue after removing the stimuli by several washings suggests that colonic tissue is not damaged. Volumes of cell extracts from *Bifidobacterium*, *Lactobacillus* and *Streptococcus* inducing the relaxation response correspond to bacterial titer values that are in the range of the physiological concentrations measured in colon[19].

Since *B. infantis* has been shown to colonize the human gut more efficiently than the other VSL#3 bacterial strains[22], we used this strain to further investigate the components of probiotic bacteria involved in the guinea-pig colon relaxation. Interestingly, only the cytoplasmatic fraction of the cell showed a pronounced colon relaxation activity. The bacterial cytoplasmic fraction may well have physiological implications, because its specific components reach the intestinal lumen as metabolites actively secreted by the viable bacteria colonizing the intestinal epithelium. In addition, the entire bacterial cytoplasm is released in the gut lumen following cell lysis. Further, subdivision of the bacterial cytoplasm allowed us to demonstrate that the

non-proteinaceous cytoplasmatic portion was responsible for the colon relaxation, whereas protein factors had no effect on proximal colon relaxation. This result has been confirmed by challenging the colon tissue with bacterial cytoplasmatic fractions digested with proteinase K.

Since the cytoplasmatic fraction deprived of the protein component contains significant amounts of genomic DNA, which in prokaryotic microorganisms is not confined to a nucleus, the proximal colon segment was tested with the bacterial genomic DNA. The DNA preparation extracted from *B. infantis* did not cause any changes in colonic motility, indicating that other unidentified non-proteinaceous factors may be involved.

In conclusion, this is the first study performed in isolated tissues that deals with the effects of probiotic bacteria on intestinal motility. Our results suggest that *Bifidobacterium*, *Lactobacillus* and *Streptococcus* strains in VSL#3 mediate proximal relaxation activity. This could be one of the possible mechanisms of action by which probiotic bacteria exert their effects in ameliorating diarrhea by reducing stool frequency and restoring a disturbed microbial balance. Based on these data, further studies to elucidate the implication of a large array of receptor families and subtypes known to affect gut function and to characterize the non-proteinaceous bacterial molecules involved in intestinal motility are warranted.

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BASIC RESEARCH

Increased DNA binding activity of NF-κB, STAT-3, SMAD3 and AP-1 in acutely damaged liver

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Abstract

AIM: To investigate the role of genes and kinetics of specific transcription factors in liver regeneration, and to analyze the gene expression and the activity of some molecules crucially involved in hepatic regeneration.

METHODS: USING gel-shift assay and RT-PCR, transcription factors, such as NF-κB, STAT-3, SMAD3 and AP-1, and gene expression of inducible nitric oxide synthase (iNOS), hepatocyte growth factor (HGF) and c-met were analyzed in an animal model of chemically induced hepatectomy.

RESULTS: Gene expression of HGF and its receptor c-met peaked at 3 h and 24 h after acute CCl_4 intoxication. iNOS expression was only observed from 6 to 48 h. Transcriptional factor NF-κB had an early activation at 30 min after acute liver damage. STAT-3 peaked 3 h post-intoxication, while AP-1 displayed a peak of activation at 48 h. SMAD3 showed a high activity at all analyzed times.

CONCLUSION: TNF-α and IL-6 play a central role in hepatic regeneration. These two molecules are responsible for triggering the cascade of events and switch-on of genes involved in cell proliferation, such as growth factors, kinases and cyclins which are direct participants of cell proliferation.

Key words: Hepatic regeneration; Transcription factor; Gene regulation

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INTRODUCTION

The liver is a unique organ with with ability to regulate its growth. This capacity can be altered at various different conditions like tissue dismissal and cell loss caused by chemical or viruses[1]. Specific molecules are involved in the molecular events originated from these processes. One of these molecules is IL-6 which is an important factor for liver regeneration and repair after injury[2]. IL-6-deficient mice fail in regenerating its hepatic gland presenting liver necrosis, functional failure, blunted DNA response in hepatocytes, absence of STAT-3 and NF-κB activation, and selective dysfunction in AP-1, c-myc and cyclin D1 gene expression[3]. IL-6 signals via STAT-3, and STAT-3 activation increase in an IL-6-dependent manner post-hepatectomy (PH) and post acute CCl_4 intoxication peaking at 2 h[4,5] and returning to basal levels at 12 h[6]. IL-6 is also an activator of AP-1 expression in liver regeneration[3]. AP-1 and STAT-3 act in a synergistic fashion enhancing transcription[7]. In our previous study IL-6 was strictly detected only at 24 h after acute CCl_4 intoxication. We could not detect IL-6 mRNA in rats intoxicated with turpentine, indicating the need of the presence of acute phase response for hepatocyte proliferation[8]. On the other hand, TNF-α has also been shown as a major effector of signal pathways of liver regeneration[9]. Several lines of evidence invoke the role of TNF-α in the regulation of IL-6 secretion through a previous induction of NF-κB[10]. Our previous results showed that induction of TNF-α gene expression takes place as early as 6 h, peaking at 48 h post-acute CCl_4 intoxication and this expression might induce IL-6 production[8]. In correlation with our results, others have shown that TNF-α activates NF-κB in many cells within 30 min after intra-peritoneal injection[11]. TNF-α and IL-6 also induce iNOS transcription through NF-κB activity[12] which occurs principally in hepatocytes due to NO production,

and is detected in these cells just after partial hepatectomy and before cell proliferation[13]. HGF, the major growth factor involved in hepatocyte proliferation, signals through its receptor c-met, a transmembrane tyrosine kinase protein product of the proto-oncogene with the same name[14]. HGF is produced by hepatic stellate cells (HSCs) and acts on cultured hepatocytes in a paracrine manner as a potent mitogen[14,15]. HGF production is induced in animals by partial hepatectomy and hepato-toxin[16], detecting the mature form in significant levels[17]. The results in animal models correlate with the elevated levels of serum HGF in patients with hepatic disorders[18]. In this study, we aimed to elucidate the kinetic of activation of several transcription factors and molecular mechanisms involved in hepatic regeneration in an animal model. Furthermore, we shed light on how these transcription factors are involved in the resolution of this process. The early activation of NF-κB, STAT-3 and AP-1 along with the expression of iNOS, HGF and c-met observed in this study suggested that induction of events like production of TNF-α, IL-6, HGF and some proteins are involved in cell proliferation. Knowledge obtained regarding activation of these transcription factors might enable us to propose new pharmacological strategies of treatment for induction of hepatic regeneration in some cases of cirrhosis.

MATERIALS AND METHODS

Animals

Forty male Wistar rats (Charles Rivers Inc., Boston, MA), weighting 200 g, were used in this study and housed according to the principles and procedures outlined by the National Institute of Health's Guide for the Care and Use of Laboratory Animals. For acute intoxication experiments, five rats for each time point were intragastrically administered a single dose of a mixture 1:1 (v/v) of CCl_4 (Merck Company, Darmstadt, Germany) and mineral oil (Sigma Chemical Company, St Louis MO, USA) at 5 mL/kg of body weight. Control animals were administrated a same volume of vehicle. Animals were sacrificed at 0.5, 1, 3, 6, 12, 24, and 48 h after CCl_4 intoxication. Livers were removed, immediately frozen in CO_2-acetone and stored at -70° until use.

Extraction and quantification of RNA

Isolation of total RNA from rat livers was carried out according to the modified method described by Chomczynski *et al*[19]. Briefly, liver tissue was taken from three different lobes to obtain a representative sample and homogenized using a Polytron System (Brinkmann, Switzerland) in the presence of Trizol (Invitrogen). Chloroform was added, the aqueous phase was obtained and the RNA was precipitated from the aqueous phase by isopropanol at 4℃ overnight. Quantity and intactness of RNA were routinely tested by determining absorbance (*A*) at 260/280 and ethidium bromide fluorescence of RNA electrophoresis on 10 g/L formaldehyde-containing agarose gels.

Analysis of iNOS, c-met and HGF gene expression

Expressions of iNOS, c-met and HGF gene were detected using RT-PCR as previously described[8]. We amplified the target genes iNOS, c-met, HGF and the constitutive gene HPRT in different reaction tubes. RNA from liver samples was isolated with Trizol and 2 g of total RNA was reverse transcribed into complementary DNA (cDNA) using 0.05 mol/L Tris-HCl (pH 8.3), 0.04 mol/L KCl, 0.007 mol/L $MgCl_2$ buffer containing 0.05 g/L random hexamers (Invitrogen), 0.001 mol/L dNTPs mix (Invitrogen), 50 U/L RNase inhibitor and 400 U of Moloney murine leukemia virus reverse transcriptase (M-MLV) (Invitrogen). Samples were incubated for 10 min at 25℃ and then for 60 min at 37℃. Reverse transcriptase was further inactivated by heating the sample tubes at 95℃ for 10 min. The cDNAs were used to perform PCR reaction according to the optimal amplification conditions for each gene. Amplification was performed in a PCR buffer of 0.05 mol/L Tris-HCl (pH 9.0) and 0.05 mol/L NaCl containing a mixture of 1×10^{-4} mol/L dNTPs and 1 unit of Taq DNA polymerase (Invitrogen). Amplification reactions were overlaid with light mineral oil and held at 94℃ for "hot-start" PCR for 3 min and then run in an automated thermal cycler for different number of cycles and incubation temperatures according to each gene. Each PCR reaction was repeated at least in triplicate. Annealing temperature, number of cycles and primer sequence for each gene are shown in Table 1.

Nuclear extract isolation

Isolation of nuclear proteins was carried out according to the methods described by Andrews *et al*[20], with a few modifications. Briefly, 1 g of liver from CCl_4-treated and controls rats was homogenized in 5×10^{-4} L of buffer A (0.01 mol/L Hepes-KOH (pH 7.9), 250 g/L glycerol, 0.420 mol/L NaCl, 0.0015 mol/L $MgCl_2$, 2×10^{-4} mol/L EDTA, 5×10^{-4} DTT, 2×10^{-4} PMSF) to disrupt extracellular matrix and cellular membranes. Homogenates were centrifuged at 1200 r/min for 10 s at 4℃. The pellet was resuspended in 2.5×10^{-4} L of buffer C (0.02 mol/L Hepes-KOH (pH 7.9), 250 g/L glycerol, 0.42 mol/L NaCl, 15×10^{-4} mol/L $MgCl_2$, 2×10^{-4} mol/L EDTA, 5×10^{-4} mol/L DTT, 2×10^{-4} mol/L PMSF), homogenized and incubated at 4℃ for 20 min. Cellular debris was removed by centrifugation at 4℃for 2 min. Supernatant fraction containing DNA binding proteins was recollected and quantified as described by Bradford[21]. Supernatant was stored at -70℃ in aliquots until use.

Gel mobility shift assays

Electrophoretic mobility shift assay (EMSA) was performed as described elsewhere[3]. Binding reactions were prepared using 2 g of nuclear extracts from either acutely damaged rat livers or from control animals. Additionally, 100 000 cpm of radioactive probe and 1.2 g of poly (DI-DC) as a non-specific DNA competitor were included in the binding reactions. Mixtures were incubated for 30 min at room temperature in binding buffer containing 0.01 mol/L Hepes (pH 7.5), 0.05 mol/L NaCl, 0.001 mol/L EDTA and 100 g/L glycerol. For competition assays, a 100-fold excess of unlabeled probe was added to the reactions concomitantly with the hot probe. For supershift experiments, 1 mL of antibody against NF-κB,

Table 1 Oligonucleotide sequences used for PCR amplification

Gene	Sequence	Annealing temperature	Cycles	Size (bp)
HPRT	5′ TCC CAG CGT CGT GAT TAG TG 3′	60℃	30	618
	5′ GGC TTT TCC ACT TTC GCT GA 3′			
iNOS	5′ TAG AGG AAC ATC TGG CCA GG 3′	58℃	25	255
	5′ TGG CCG ACC TGA TGT TGC CA 3′			
c-MET	5′ CAG TGA TGA TCT CAA TGG GCA AT 3′	60℃	28	725
	5′ AAT GCC CTC TTC CTA TGA CTT C 3′			
HGF	5′ AGC TCA GAA CCG ACC GGC TTG CAA CAG GAT3′	60℃	27	618
	5′ TTA CCA ATG ATG CAA TTT CTA ATA TAG TCT3′			

Table 2 Oligonucleotide sequences used for EMSA

Transcription factor	Sequence
NF-κB	5′ AGT TGA **GGG GAC TTT CCC** AGG C 3′
	3′ TCA ACT **CCC CTG AAA GGG** TCC G 5′
STAT-3	5′ GAT CC**T TCT GGG AA**T TCC 3′
	3′ CTA GG**A AGA TCC TT**A AGG 5′
SMAD-3	5′ TCG AGA GC **CAGA** CAA AAA GC **CAGA** CAT TTA GC **CAGA** CAC 3′
	3′ AGC TCT CG **GTCT** GTT TTT CG **GTCT** GTA AAT CG **GTCT** GTG 5′
AP-1	5′ GAT CGA **TGA CTC A**GA GGA AAA 3′
	3′ CTA GCT **ACT GAG T**CT CCT TTT 5′

Bold and underlined letters denote specific consensus DNA-binding sequences.

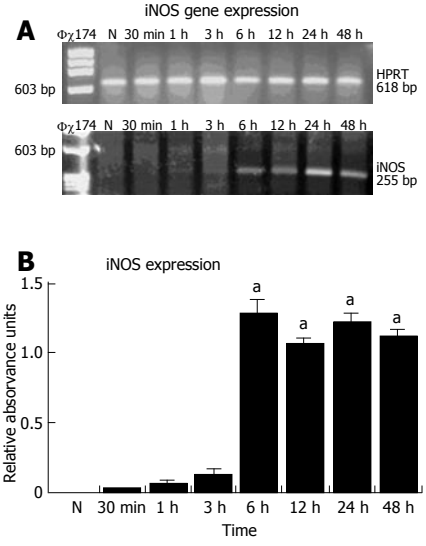

Figure 1 Semiquantitative RT-PCR analysis for iNOS expression. **A:** PCR products analyzed by agarose electrophoresis; **B**: normalized values of iNOS expression with respect to the housekeeping gene HPRT in all analyzed groups of three different PCR reactions performed.

STAT-3, AP-1 or SMAD3 (Santa Cruz Biotechnology) was incubated with the reaction mixture for 1 h at room temperature before regular incubation. The reactions were analyzed on 5% acrylamide non-denaturing gels in 0.5 × Tris-borate-EDTA buffer, dried and exposed. Intensity of each band, as the measure of DNA binding activity, was assessed by densitometric scanning Kodak ID 3.6 program. For gel retardation experiments, single-stranded oligonucleotides were obtained from Sigma and annealed

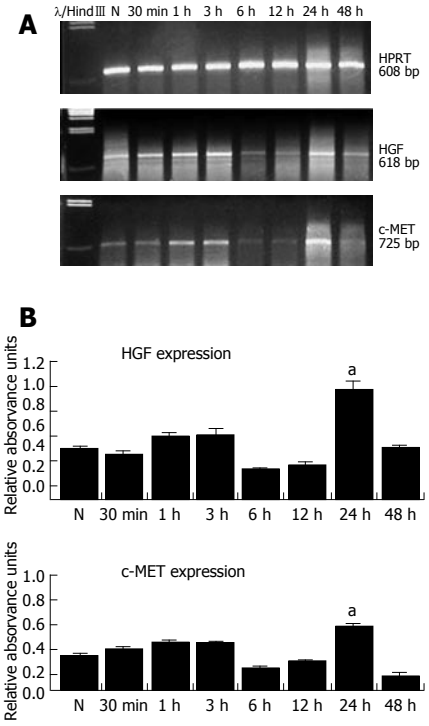

Figure 2 Semiquantitative RT-PCR analysis for HGF and c-met expressions. **A**: PCR products analyzed by agarose electrophoresis; **B**: Normalized values of HGF and c-met expression with respect to the housekeeping gene HPRT in all analyzed groups of three different PCR reactions performed.

in water. For annealing of complementary oligonucleotide pairs, 5 μg of each single-strand oligonucleotide was adjusted to a final volume of 5×10^{-5} L and placed on a heating blocker at 95℃ for 5 min. Then the blocker was turned off and left to reach room temperature. Double-strand probe end labeling was performed using T4 polynucleotide kinase (Gibco) in the presence of (γ-32P) ATP. Each gel-shift experiment was performed in triplicate. Probe sequences for NF-κB, STAT-3, SMAD3 and AP-1 are shown in Table 2.

Statistical analysis

Results were expressed as mean ± SD. Student's *t* test was used to analyze the data. $P < 0.05$ was considered statistically significant.

RESULTS

After normalization against the housekeeping gene HPRT, iNOS expression was detected at 6 h after acute liver damage and continued up to 48 h, being statistically different at these times ($P < 0.05$). However, iNOS expression was not detected before 6 h (Figure 1).

The hepatocyte growth factor (HGF) and its cognate receptor c-met mRNAs after CCl_4 acute intoxication are presented in Figure 2. Two peaks of gene expression were observed in both genes: one at 3 h and another at 24 h where significant difference was seen only at 24 h ($P < 0.05$).

It has been shown that transcription factors are

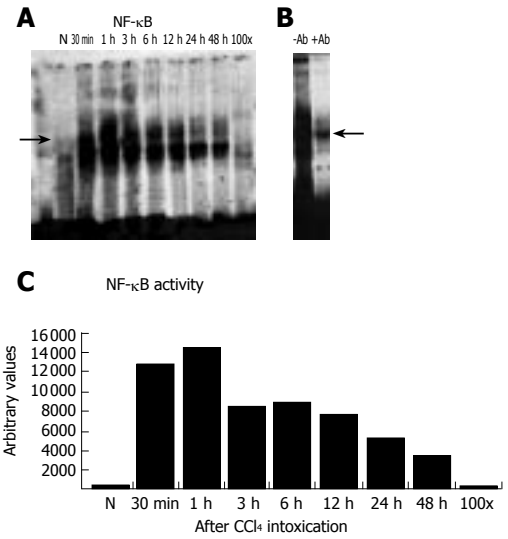

Figure 3 Binding activity of NF-κB in acute liver damage. Wistar rats were intoxicated acutely with CCl_4 and then sacrificed at different time points. Nuclear proteins were extracted from the livers and Gel-shift assay was performed for NF-κB transcription factor. **A**: Binding DNA activity of NF-κB after CCl_4 intoxication; **B**: supershift assay using 1-h sample, 1 μL of polyclonal antibody against p65 subunit was added and incubated for 1 h before usual EMSA assay; **C**: densitometric analysis of results of EMSA assay.

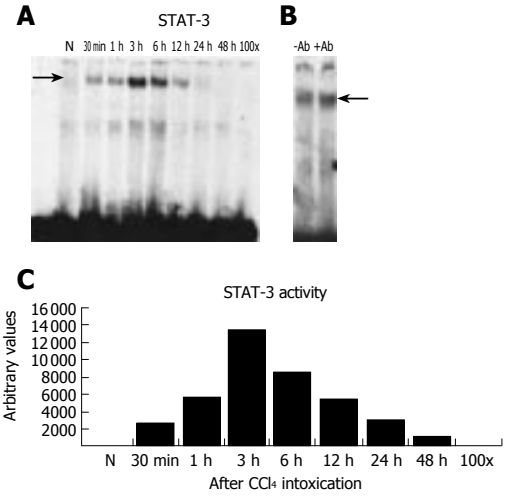

Figure 4 Binding activity of STAT-3 in acute liver damage**.** Wistar rats were intoxicated acutely with CCl_4 and then sacrificed at different time points. Nuclear proteins were extracted from the livers and Gel-shift assay was performed for STAT-3. **A**: Binding DNA activity of STAT-3 after CCl_4 intoxication; **B**: supershift assay similar using 6-h sample; **C**: densitometric analysis of results of EMSA assay.

activated during hepatic regeneration in different hepatectomy models[9,22,23]. To determine the role and kinetic of activation of transcription factors critically involved in hepatic regeneration after acute CCl_4 injury, we performed gel-shift assays to evaluate the binding activity of NF-kB, STAT-3, SMAD-3 and AP-1 on DNA probes containing consensus sequences. We performed a chronological analysis between 0.5 h and 48 h after liver damage, since it has been reported by others and us, that the inflammatory process has declined and cell proliferation has concluded by this time[24]. Transcriptional

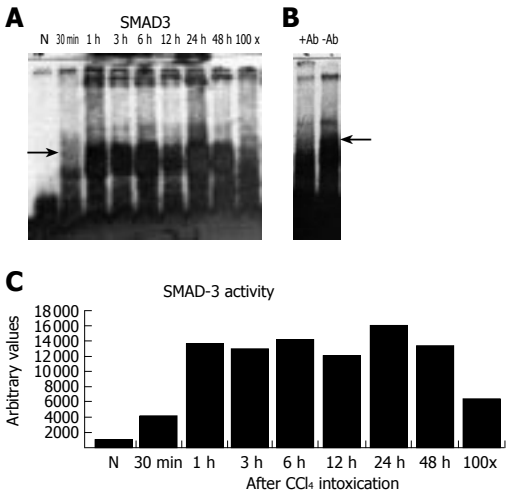

Figure 5 Binding activity of SMAD3 in acute liver damage. Gel-shift and supershift assays were performed to analyze SMAD3 DNA binding activity. **A**: Binding DNA activity of SMAD3 after CCl_4 intoxication; **B**: supershift assay using 24-h sample; **C**: densitometric analysis of results of EMSA assay.

factor NF-κB presented strong DNA binding activity from 30 min after CCl_4-induced injury, peaking at 1 h and decreasing thereafter. Nevertheless, 48 h after CCl_4-induced injury, NF-κB activity did not return to normal levels (Figure 3A). To confirm the specific binding activity of NF-κB, we performed supershift assay using specific anti-NF-κB antibodies. The use of a polyclonal antibody against the p65 subunit of NF-κB decreased the binding of this transcription factor to the labeled probe, suggesting the binding of the antibody to the DNA binding site of the transcription factor (Figure 3B). Furthermore, almost complete elimination of the radiolabeled band, when a 100-fold cold DNA probe was used, confirmed the specificity of our results. These results were confirmed by densitometric analysis (Figure 3C).

With respect to STAT-3 activity, our results showed an increase at 30 min after intoxication, presenting the maximum activity at 3 h, and then decreasing and disappearing completely by 48 h (Figure 4A). We also made a supershift assay for this transcription factor obtaining a clear DNA binding reduction. The data suggest that, in our experimental conditions, this antibody preferentially binds to the DNA binding site on the transcriptional factor, thus hindering formation of STAT-3-DNA consensus site complex. This piece of data verifies that the results corresponded to this transcription factor (Figure 4B). These results were confirmed by densitometric analysis (Figure 4C).

On the other hand, SMAD3 showed a strong binding activity in this animal model at all analyzed times, as was confirmed by densitometric analysis at 1 h after CCl_4 intoxication until 48 h (Figures 5A and C). The specificity of the binding activity was analyzed by supershift assay (Figure 5B).

The transcription factor AP-1 showed a basal activity in normal animals. This activity increased as early as 30 min after CCl_4-induced injury and increased progressively showing the maximum peak in the last analyzed time in

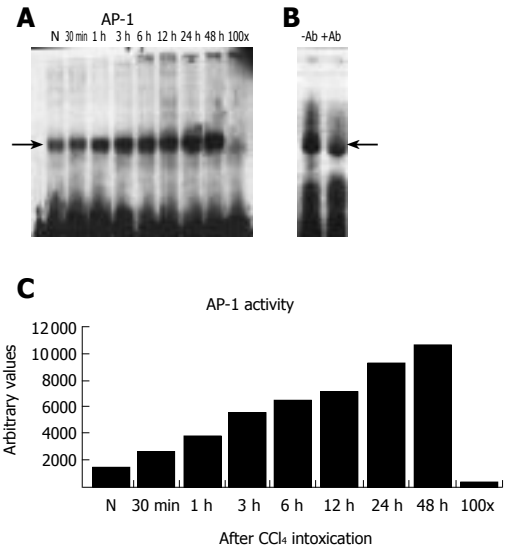

Figure 6 Binding activity of AP-1 in acute liver damage. EMSA and supershift assays, similar to Figures 3 and 4, were performed to analyze AP-1 activity in the same samples. **A**: Binding DNA activity of AP-1 after CCl_4 intoxication; **B**: supershift assay using 24-h sample; **C**: densitometric analysis of results of EMSA assay.

this study, 48 h after intoxication. Figures 6A-B clearly show the specificity of AP-1 DNA-binding activity when an excess of cold probe and specific anti-AP-1 antibody were used to override the binding of nuclear factor to the radiolabeled DNA-consensus sequence. These results were confirmed by densitometric analysis (Figure 6C).

DISCUSSION

The increase in DNA binding activity of the transcription factors NF-κB, STAT-3 and AP-1 analyzed in this study indicates that hepatic regeneration process in response to CCl_4-induced acute liver damage requires the switch on and the switch off of many genes. These genes include cytokines, growth factors, kinases and cyclins which regulate cell cycle and induce hepatocyte proliferation. Our previous study demonstrated that expression of IL-6 is strongly associated with hepatic regeneration[8]. We observed IL-6 gene expression in acute liver damage between 6 and 24 h, and disappeared thereafter. Other pro-inflammatory cytokines like IL-1β and TNF-α also show strong expression around 24 h after liver damage[24,25]. Because NF-κB is required for TNF-α and iNOS production[12], our results suggest that the strong inflammatory response present in CCl_4-intoxicated animals causes oxidative stress manifested as an increase in NF-κB activity which induces iNOS expression. Although the maximum activity of NF-κB was detected at 1 h post-CCl_4 intoxication, and iNOS expression was not observed before 6 h, suggesting that some hours are required for iNOS RNAm to be detected. This inflammation is accompanied with damage and death followed by hepatocyte proliferation where TNF-α plays an important role[26]. The activation of NF-κB, STAT-3, SMAD3 and AP-1 allows their migration to the nucleus, where they can bind to their consensus sequence and induce the expression of several genes involved in inflammation and cell proliferation[27]. TNF-α promoter contains multiple binding sites for NF-κB being a vital component for its expression and a ubiquitous oxidative stress-sensitive transcription factor[28]. NF-κB is found in almost all cell types, including hepatocytes and non-parenchymal cells[29]. In absence of NF-κB, TNF-α functions as an apoptotic agent in liver development. The NF-κB inhibitor (Iκ-B) degradation is enhanced by reactive oxygen species that can be generated by TNF and many other agents and cellular processes[30].

Evidence indicates that blockade of NF-κB in the regenerating liver by expression of NF-κB super-repressor in an adenovirus vector leads to apoptosis after the cells have replicated their DNA[31]. Similarly, introduction of I-κB in an adenovirus vector after partial hepatectomy in mice results in increased liver injury and decreased hepatocyte cell proliferation.

On the other hand, transcription factor STAT-3 is also activated after partial hepatectomy but its activation is delayed compared to NF-κB. STAT-3 becomes activated mainly by IL-6 type cytokines[32]. Binding of IL-6 causes dimerization of the receptor, activation of tyrosine kinases which phosphorylate gp130 and create docking sites for STAT-3 binding. IL-6/STAT-3 signaling pathway is involved in cell proliferation through the induction of cyclins D1, D2, D3, A, cdc25A and concomitant down-regulation of cyclin-dependent kinase (cdk) inhibitors p21 and p27[33,34]. p53, mdm2, p21, cyclins and cdks genes are also activated. STAT-3 activation observed in this study is in agreement with previous reports which demonstrated binding activity as early as 30 min using partial hepatectomy model to induce liver regeneration[1,23]. SMAD3 have been found to be involved in hepatic stellate cell activation and collagen production after liver damage[34], since high level of transcription factor SMAD3 could be detected even after 48 h of CCl_4 intoxication. These results suggest that liver damage caused by CCl_4 intoxication has not resolved at this time and some genes involved in the damage resolution activated by SMAD3 like TGF-α and collagen I are being expressed.

The role of AP-1 in the expression of molecule participants in cell proliferation, such as c-myc, D1 cyclin and cell growth factors, have been reported[23]. The results obtained with AP-1 support, in fact, the role of AP-1 in hepatic regeneration. In this study, we observed a higher activity of AP-1 and also the maximum hepatocyte proliferation between 24 h and 48 h after CCl_4-induced liver damage, which are in agreement with previous studies[35,36]. The initiation step called "priming step" appears to be mediated by TNF-α and IL-6 and their downstream pathways involving activation of NF-κB, STAT-3 and AP-1[1,37]. Activation of these transcription factors leads the progression to G1 phase of the cell cycle[33]. "Priming" of hepatocytes induces them to respond to extra- and intra-hepatic growth factors, such as epidermal growth factor (EGF), transforming growth factor-β (TGF-β) and HGF[1]. In this study, we found HGF gene expression and its receptor c-met peaking at 1 and 24 h after the liver damage. These results allow to us think that HGF expression could be induced by two

different pathways involving two different molecules, first TNF-α and then IL-6. Since HGF strongly stimulates DNA synthesis in damaged hepatocytes, an increase in HGF and its receptor mRNA expression suggests that liver regeneration is taking place[15,17].

TNF-α signals through two distinct receptors: TNFR-1 and TNFR-2[38]. Mice lacking functional TNFR-2 show completely normal DNA replication after hepatectomy and CCl_4 treatment. In contrast, lack of signaling through TNFR-1 greatly inhibites DNA replication after partial hepatectomy and cause significant mortality 24-40 h after the operation[39] In TNFR-1-knockout mice, activation of NF-κB and STAT-3 is inhibited and AP-1 activation is decreased. The signal transduction pathway starting from TNF-α required for liver regeneration involves TNFR-1 with NF-κB activation. The sequence of events proposed for liver regeneration after CCl_4-intoxicated acute liver damage is similar to that observed with different animal models like partial hepatectomy. However, the time of activation seems to be more delayed. The sequence of these events is as follows: TNF-α binds to TNFR-1 and induces activation of NF-κB, NF-κB binds to IL-6 promoter and the protein is produced. IL-6 activates STAT-3, which in turn activates AP-1. AP-1 participates in expression of the genes involved in hepatocyte proliferation, such as D1 cyclin, c-myc and kinases[7,9].

In conclusion, TNF-α and IL-6 are responsible for triggering the cascade of events and switch-on of genes involved in cell proliferation, such as growth factors, kinases and cyclins which are direct participants of cell proliferation.

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CLINICAL RESEARCH

Reduced expression of Ca^{2+}-regulating proteins in the upper gastrointestinal tract of patients with achalasia

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Abstract

AIM: To compare expression of Ca^{2+}-regulating proteins in upper gastrointestinal (GI) tract of achalasia patients and healthy volunteers and to elucidate their role in achalasia.

METHODS: Sarcoplasmic reticulum Ca^{2+} ATPase (SERCA) isoforms 2a and 2b, phospholamban (PLB), calsequestrin (CSQ), and calreticulin (CRT) were assessed by quantitative Western blotting in esophagus and heart of rats, rabbits, and humans. Furthermore, expression profiles of these proteins in biopsies of lower esophageal sphincter and esophagus from patients with achalasia and healthy volunteers were analyzed.

RESULTS: SERCA 2a protein expression was much higher in human heart (cardiac ventricle) compared to esophagus. However, SERCA 2b was expressed predominantly in the esophagus. The highest CRT expression was noted in the human esophagus, while PLB, although highly expressed in the heart, was below our detection limit in upper GI tissue. Compared to healthy controls, CSQ and CRT expression in lower esophageal sphincter and distal esophageal body were significantly reduced in patients with achalasia ($P < 0.05$).

CONCLUSION: PLB in the human esophagus might be of lesser importance for regulation of SERCA than in heart. Lower expression of Ca^{2+} storage proteins (CSQ and CRT) might contribute to increased lower esophageal sphincter pressure in achalasia, possibly by increasing free intracellular Ca^{2+}.

Key words: Esophageal and gastric motility; Esophagus; Calsequestrin; Calreticulin

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INTRODUCTION

Intracellular Ca^{2+} regulates contractility in striated, smooth, and heart muscle. In the heart, expression of the most important proteins for intracellular Ca^{2+} homeostasis has been studied extensively. β-adrenoceptor agonists elevate Ca^{2+} by activation of cAMP-dependent protein kinase (PKA) and subsequent phosphorylation of regulatory proteins. The predominant protein phosphorylated in cardiac sarcoplasmic reticulum (SR) by PKA is phospholamban (PLB). The best characterized function of phospholamban is the regulation of the activity of the SR or endoplasmatic reticulum (ER) Ca^{2+}-pump (SERCA). In isolated guinea-pig hearts, β-adrenergic stimulation leads to phosphorylation of PLB and, at the same time, Ca^{2+}-uptake into the SR is increased[1]. PLB in its dephosphorylated state lowers the Ca^{2+} affinity of SERCA, while phosphorylation of PLB reduces the affinity of SERCA for Ca^{2+}. Therefore, PLB phosphorylation increases the rate of Ca^{2+}-transport into the SR. As a consequence, the rate of cardiac relaxation as well as contraction increase[2,3]. SERCA itself is coded by three genes in mammalians: SERCA 1, 2, and 3[4]. SERCA 1 usually is expressed in fast-twitch skeletal muscle and SERCA 3 in non-muscle cells. For SERCA 2, two splice variants are known: SERCA 2a and SERCA 2b, the latter containing 49 additional amino acids at the carboxyterminal end[5]. In the heart SERCA 2a

is the predominant isoform, whereas SERCA 2b is found mainly in smooth muscle, but smaller amounts have been recognized also in other tissues like heart[6].

Intracellular rapidly exchanging Ca^{2+} stores provide the possibility for release, uptake, and storage of Ca^{2+} transported into SR by SERCA. Ca^{2+}-binding proteins with high capacity and low affinity for Ca^{2+}-binding permit rapid Ca^{2+} release. At least two families of these Ca^{2+}-binding proteins have been described, namely calsequestrin (CSQ) and calreticulin (CRT). CSQ is found in skeletal muscle and heart, while CRT is more widely distributed[7,8]. CSQ is coded by two genes, the skeletal muscle isoform (CSQ1) coding for a 62-ku protein, and the cardiac isoform (CSQ2) coding for a 51-ku protein[9,10]. Differences within the amino acid sequences might be functionally relevant, because the deduced amino acid sequence of cardiac calsequestrin is consistent with its ability to bind larger amounts of Ca^{2+}[10]. In contrast, CRT is coded by one gene and splice variants have not yet been reported[11].

In heart, regulation of SERCA2a activity by expression and phosphorylation of PLB is well established, and disturbances of this system might be involved in pathophysiological events leading to heart failure[3,12,13]. Indeed, targeted over-expression of CSQ or CRT in the heart of transgenic mice led to cardiac hypertrophy, dilated cardiomyopathy, and heart failure[14-16]. However, expression, function, and pathophysiological role of Ca^{2+}-processing proteins in the human gastrointestinal (GI) tract and their alterations in diseased states have not been examined before.

There are few diseases of the GI tract where altered Ca^{2+} homeostasis might be involved. One of the possible entities is achalasia. Achalasia is a rare motility disorder characterized by increased pressure of the lower esophageal sphincter (LES) and simultaneous contractions of the esophageal body (EB) leading to impaired swallowing. There is evidence that gastric motility is also impaired in achalasia[17,18]. Common treatments of achalasia are invasive procedures like pneumatic dilatation and laparoscopic myotomy. For medical therapy, smooth muscle relaxants as nitrates and Ca^{2+}-channel blockers of the dihydropyridine-type are effective in the treatment of achalasia[19]. Hence, we tested the hypothesis that the expression of SR-proteins involved in Ca^{2+} handling in the GI tract is altered in achalasia in humans.

MATERIALS AND METHODS

Animals

Male Wistar rats (body weight, 150-250 g) and rabbits which had fasted overnight were sacrificed. Organs (esophagus, heart) were prepared and immediately frozen in liquid nitrogen.

Human cardiac tissue

Samples were taken from left ventricles of non-failing hearts which were obtained from prospective organ donors whose hearts could not be used. The study was performed in accordance with the guidelines from the Local Ethics Committee.

Patients

Manometrically proven 9 patients with achalasia (age, 19-52 years), and 6 healthy controls (age, 22-49 years) participated in the study. None of the control group had a history of GI disease or complained of any symptom of a GI disease. All patients underwent gastroduodenoscopy and ^{13}C-urea breath test to exclude gastroduodenal disease or infection with *H pylori*. During endoscopy, biopsies of esophagus and LES were taken. Biopsies were immediately frozen in liquid nitrogen. The study had been approved by the Local Ethics Committee, and all patients gave written informed consent.

Esophageal manometry

Patients were fasted overnight before manometric examination was performed using a low-compliance, water-perfused system. The Arndorfer catheter-with four side-holes oriented radially, 0.5 cm apart, located distally for LES (lower esophageal sphincter) examination and four more orifices located every 5 cm above the distal ones thus allowing for the assessment of the motility pattern in the middle and distal part of the EB (esophageal body)-was attached to force transducers. The recorded signals were amplified (Polygraph Ⅷ, Synectics, Stockholm, Sweden) and stored for further analysis with a specially designed software (Gastrosoft, Irving, Texas, USA). The motility patterns of distal esophagus were examined with (wet swallows) and without (dry swallows) swallowing of 5 mL of water bolus and expressed as mean values of the amplitude (mm Hg), duration (s) and propagation (cm/s) as calculated for five wet and five dry swallows. The LES resting tone was assessed by pull-through technique and the mean value, expressed in mm Hg above the mean intragastric pressure, was calculated from three consecutive measurements. The LES relaxation was measured in response to 5 mL of water bolus, while the distal catheter orifices were located in the LES high-pressure zone and results were documented as complete or incomplete relaxation of the LES.

SDS-PAGE and Western blot analysis

Gel electrophoresis was performed according to the method described by Laemmli[20]. Briefly, samples were homogenized in 10 mmol $NaHCO_3$/50 g/L SDS using a microdismembrator (Braun, Melsungen, Germany) and the protein content of homogenates was measured according to Lowry *et al*[21]. After SDS polyacrylamide gel electrophoresis, the separated proteins were transferred onto nitrocellulose membranes (Schleicher and Schüll, Dassel, Germany) as described previously[22]. For immunostaining, the following primary antibodies were used: monoclonal anti-PLB (2D12), monoclonal anti-SERCA 2a (2A7-A1), polyclonal anti-CSQ (all kindly provided by L.R. Jones, Krannert Institute of Cardiology, Indianapolis, USA), polyclonal anti-SERCA 2b (kindly provided by L. Raeymaekers and Wuytack, University of Leiden, Belgium), and polyclonal anti-calreticulin (Alexis, Grünberg, Germany). Thereafter, bound primary antibodies were detected with [^{125}I]-labeled protein A (ICN, Meckenheim, Germany). Visualization and

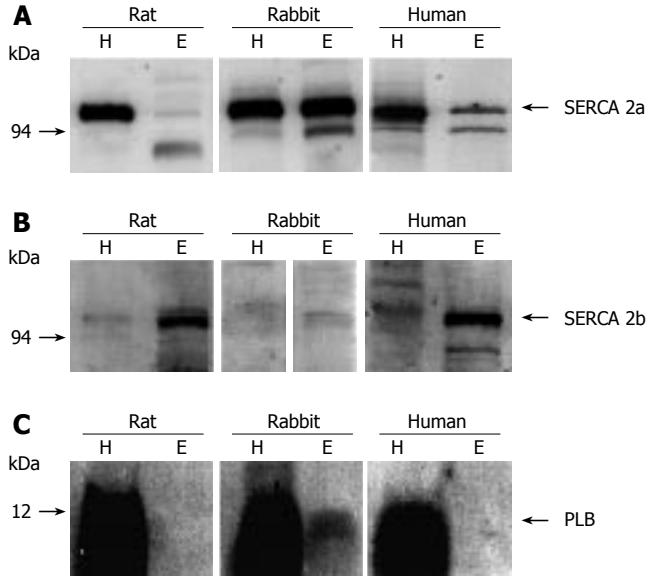

Figure 1 Protein expression of SERCA 2a (**A**), 2b (**B**), and PLB (**C**) in esophageal and ventricular homogenates of rat, rabbit, and human. Protein loading for esophagus (100 μg) was 5 times higher than for heart (20 μg). For documentation of PLB, sensitivity of the PhosphorImager was optimized for detection of very weak signals. Therefore, signals in heart samples are overexposed. H: heart; E: esophagus.

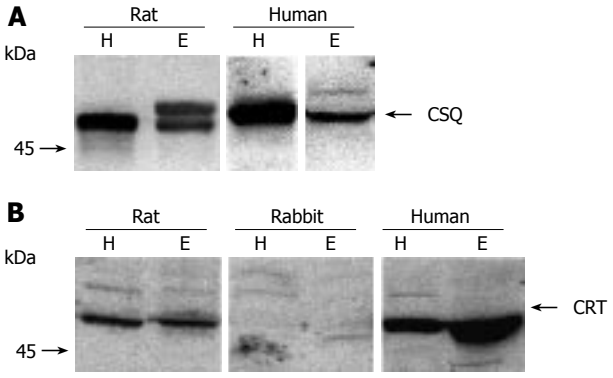

Figure 2 Protein expression of CSQ (**A**) and CRT (**B**) in esophageal and ventricular homogenates of rat, rabbit, and human. Protein loading was 50 μg/lane for CSQ and 100 μg/lane for CRT. H: heart; E: esophagus.

quantification of protein bands were performed with a PhosphorImager system (Molecular Dynamics, Krefeld, Germany). Differences in protein loading were corrected by densitometric quantification of Ponceau-stained membranes.

Statistical analysis

Results were presented as mean ± SE. The significance of differences between means was evaluated using Mann-Whitney test with a confidence value at $P < 0.05$.

RESULTS

First, we studied the expression of Ca^{2+}-regulatory proteins in the upper GI tract. For comparison, we used cardiac tissues where the distribution of Ca^{2+}-regulatory proteins has been established before[23,24]. Finally, we were interested in their putative pathophysiological role in man. Of note, to our knowledge, expression of these proteins has not been studied before in the human GI tract. However, as more data on expression are available for animal tissues than for human tissues, we studied, where possible, human and animal tissues in parallel. First, we analyzed the expression of SERCA in homogenates from various tissues. Figure 1 depicts protein expression of SERCA 2a and 2b in the esophagus and heart of different species (human, rat, and rabbit). Although, only 20 μg of ventricular protein was loaded compared to 100 μg for esophagus, it is obvious that SERCA 2a protein expression was highest in the cardiac ventricle in all three species analyzed (Figure 1A). In rabbit esophagus, the SERCA 2a signal was quite pronounced, whereas in human and rat esophageal expression of SERCA 2a was hardly detectable (Figure 1A). Although, primary sequences of SERCA 2a are somewhat different between rat, rabbit, and human, these expressional differences are valid, as comparisons only were done within one species[4]. However, the SERCA 2a protein sequence expressed in esophagus and heart of a given species is identical. In rat and human, where SERCA 2a expression was barely detectable in 100 μg of esophagus protein, a strong signal was visible in 20 μg of cardiac proteins (Figure 1A). This strongly argues for tissue differences in expression between esophagus and heart at least in rat and, more importantly, in human. In contrast, SERCA 2b expression could be found predominantly in rat and human esophagus, while rabbit esophagus did contain only very low levels of SERCA 2b (Figure 1B). Only a very weak signal for SERCA 2b was detectable in heart, as expected (Figure 1B). For subsequent quantification, it was important to establish the linearity of protein detection by Western blotting. Detection of SERCA 2b in human left ventricle and esophagus as well as in rat samples was linear over a range of 50 to 200 μg of protein loaded per lane (data not shown).

Next, we compared PLB expression in rat, rabbit, and human samples (Figure 1C). Strong signals could be detected in all homogenates of cardiac ventricles, while in rat and human esophagus, expression of PLB was below our detection limit (defined as 5% over background). Only in rabbit esophagus, a weak PLB signal was detectable (Figure 1C).

Both CSQ isoforms were expressed in esophagus of human and rat (Figure 2A). In the heart, only the low molecular weight isoform was present (Figure 2A). The high molecular weight band was identified as the skeletal muscle isoform and the lower band as the cardiac isoform of CSQ, based on the literature[7].

We studied protein expression of CRT in all tissues. Expression of CRT in rat was nearly the same in esophagus and cardiac ventricle (Figure 2B). In humans, CRT expression was lower in heart than in esophagus (Figure 2B). In rabbit, CRT signal was much weaker in both tissues compared to human and rat. Conceivably this was a problem of the specificity of the antibody we used for detection of CRT.

After determination of protein expression of SERCA, PLB, CSQ, and CRT in different species, we posed the

Table 1 Ratios of CRT/CSQ and CRT/SERCA 2b in esophageal body (EB) and lower esophageal sphincter (LES) and LES pressure of achalasia patients and healthy controls (mean ± SE)

	EB		LES	
	Control (n = 6)	Achalasia (n = 9)	Control (n = 6)	Achalasia (n = 9)
Pressure (mm Hg)	ND	ND	31.2 ± 2.9	49.2 ± 3.8[a]
CRT/CSQ	0.63 ± 0.08	0.61 ± 0.04	0.88 ± 0.10	0.78 ± 0.08
CRT/SERCA 2b	1.07 ± 0.20	0.77 ± 0.10	1.47 ± 0.09	1.07 ± 0.08[a]

CRT: Calreticulin; CSQ: Calsequestrin; SERCA: Sarcoplasmic reticulum Ca^{2+} ATPase; ND: Not done. [a]$P < 0.05$ *vs* controls.

question, whether expression of these proteins might be altered during GI disease. Ca^{2+} plays an important role in GI motility. Hence, we compared SERCA 2b, CSQ, and CRT expression in healthy controls and patients with achalasia (LES pressure was elevated by 58%, Table 1). In biopsies from LES and EB, SERCA 2b expression was not obviously different between controls and patients (Figure 3). However, CSQ expression in LES and EB was found to be significantly reduced in patients with achalasia (LES reduced by 19%, and EB by 22%) (Figure 3). CRT expression was reduced by 26% in LES as well as in EB of patients with achalasia (Figure 3). CRT/CSQ ratio was not markedly different between both groups, while CRT/SERCA 2b ratio was significantly reduced by 27% in LES of achalasia patients (Table 1).

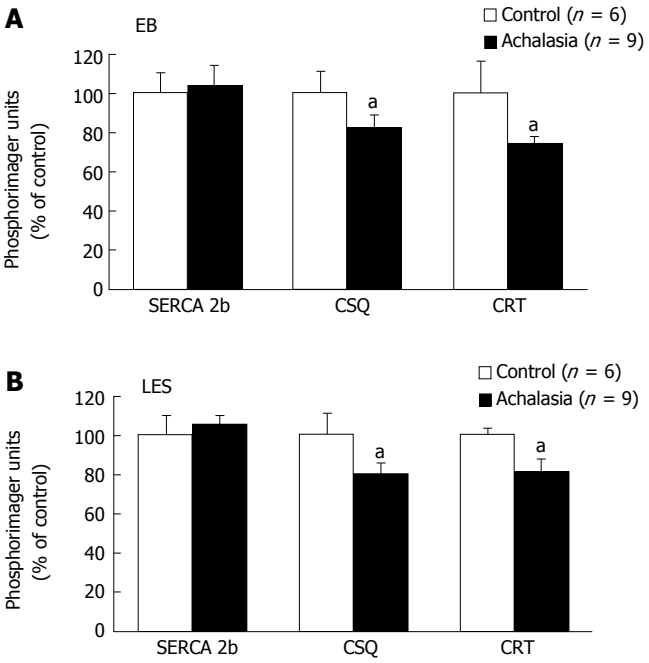

Figure 3 Protein expression of SERCA 2b, CSQ, and CRT in biopsies of esophageal body (EB, **A**) and lower esophageal sphincter (LES, **B**) from patients with achalasia or healthy controls. Protein expression was quantified using [^{125}I]-labeled protein A for detection of primary antibodies. Radioactive bands were visualized and quantified using a PhosphorImager. Data are presented as mean ± SE of n patients. [a]$P < 0.05$ *vs* control.

DISCUSSION

For phasic smooth muscles, it was shown that SR Ca^{2+} cycling can play a major role in modulating mechanical activity[25]. As achalasia, a human GI motility disorder, can be treated with drugs modulating intracellular Ca^{2+}, one can speculate that impaired Ca^{2+}-homeostasis might be involved in development or deterioration of this disease. Therefore, aim of this study was to compare protein expression of Ca^{2+}-regulating proteins in upper GI tract from different species and moreover, to ascertain if they could play a role in human diseases of the upper GI tract. In rats, as well as in humans, SERCA 2a was predominantly expressed in cardiac tissue, while expression was hardly detectable in esophageal tissue. However, rabbit esophagus contained high amounts of SERCA 2a, while SERCA 2b was not detectable in both, cardiac and esophageal, homogenates of this species. Our data concerning the relative expression of SERCA 2a and SERCA 2b in rat cardiac and esophageal homogenates are in accordance with observations by Wu *et al*[26] who reported a similar ratio of SERCA 2a/2b expression in rat heart (30:1) and esophagus (1:4) at the mRNA level. Similar distribution of SERCA 2a and 2b RNAs in rat stomach and heart have been described[27]. However, esophagus was not analyzed in this study. Another study, using the sensitive nuclease protection assay, determined a SERCA 2a/2b mRNA ratio of 20:1 in rabbit cardiac tissue, whereas in most smooth muscle and non-muscle tissues SERCA 2b was expressed predominantly[28]. Surprisingly, in rabbit esophagus, SERCA 2a accounted for 80% of SERCA 2 content[28]. These data are in accordance with our observation that in rabbit esophagus SERCA 2a expression was much more pronounced than in esophagus of rats and humans (compared to heart of the same species). Correspondingly, the SERCA 2b signal was significantly weaker in rabbit than in rat and human esophagus. Although, species specificity of the antibody (the alternatively spliced 50 amino acid carboxy terminal tail exhibits 10 differences between rabbit and man, 3 between rat and human and 10 between rabbit and human, most of these changes being conservative replacements[28]) might contribute to this phenomenon, it is conceivable that the weaker expression of SERCA 2b might be due to a lesser importance of this isoform in rabbit esophagus. In summary, there are differences in the relative expression of SERCA 2a and 2b. Hence, splicing of SERCA 2 exhibits distinct tissue and species differences. Similar results at the protein level had been published elsewhere[29]. But esophageal tissue was not analyzed so far.

In our study, expression of PLB was abundant in cardiac tissue of all species analyzed. A weak PLB signal could be obtained in rabbit esophagus. In the esophagus of rats and humans, PLB was below the detection limit. Only sparse data on PLB expression in the GI tract are available. Varying amounts of PLB mRNA and protein with a strong signal in gastric smooth muscle of pigs, but no signal in aorta were noted[30]. In canine smooth muscle cells of ileum, PLB was detected by immunogold microscopy, but only ileum, not esophagus, was analyzed[31]. Another group detected PLB in pig stomach, rat aorta and dog aorta, but not in pig aorta. They concluded from their experiments that PLB might exist in many, but not all muscle tissues[32].

However, in our experiments, we failed to detect PLB in human and rat esophagus. This has not been studied and reported before. Our observation might be due to the fact that PLB is only of minor functional importance for SERCA regulation in these tissues.

May these discrepancies have functional consequences? Some studies have addressed the question of how SERCA 2a and 2b interact with PLB in different muscle tissues. The effect of cAMP- and cGMP-dependent protein kinases on Ca^{2+} uptake of ER vesicles is smaller in smooth muscle than in cardiac tissue[33]. Using transfection experiments with COS-1 cells, a slower turnover rate for SERCA 2b was found and a higher apparent affinity for Ca^{2+} was reported for SERCA 2b compared to SERCA 2a. Ca^{2+}-affinity was decreased in both cases by co-expression with PLB[5,34]. John *et al*[35] reported similar functional differences of SERCA 2a and 2b isoforms. Additionally, they described the ability of CRT to modulate SERCA 2b-, but not SERCA 2a-activity, most likely by a binding to the additional eleventh transmembrane segment and luminal carboxy terminus of SERCA 2b. Since PLB expression in human and rat esophagus was below the detection limit, in contrast to cardiac PLB expression, esophageal PLB (at least in rats and humans) seems to be of lesser importance for regulation of SERCA than in heart. It is tempting to speculate that regulators other than PLB, possibly CRT, might control SERCA 2b and Ca^{2+}-handling in these tissues. In agreement with this speculation, we detected PLB expression only in rabbit esophagus, where a high amount of SERCA 2a, but no CRT was present, while CRT was detectable in rat and human esophagus, where SERCA 2b, but not PLB, was expressed.

In accordance with the literature, we found two isoforms of CSQ in rat, a low and high molecular weight isoform. In the heart exclusively the low molecular form was expressed, while smooth muscle tissue contained both isoforms[7]. Here we have demonstrated that human esophagus also contains both isoforms of CSQ. To the best of our knowledge, CSQ protein expression in human upper GI tract has not been described before.

CRT protein expression was found in esophagus of rat and man and, to lesser extent, in cardiac tissue of these species, while it was missing in rabbit tissue. CRT expression, although being detectable in high amounts in various tissues, has not been analyzed in human esophagus. The expression patterns, presented here, encourage the speculation that CRT might be involved in the regulation of esophageal SERCA 2b.

Finally, we found decreased expression of CSQ and CRT in LES and EB of patients with achalasia. Although, antagonists of L-type Ca^{2+}-channels like nifedipine have proven effective in treatment of achalasia, studies on free intracellular Ca^{2+} levels in achalasia have not been published. It might be speculated that decreased CRT and CSQ levels might lead to increased intracellular free Ca^{2+} and therefore to elevated LES pressure. Interestingly, over-expression of CSQ decreases the free Ca^{2+} in the cytosol of cardiac myocytes[15]. This finding may explain why contractility of CSQ-over-expressing cardiac cells was diminished compared to wild-type cells. In contrast, one can speculate that reduced expression of CSQ might be accompanied by increased free cytosolic Ca^{2+} followed by enhanced contractility. If this mechanism comes true for smooth muscle cells, reduced expression of CSQ might lead to elevated free Ca^{2+} levels and hence increased tension and therefore to achalasia in patients. Reduced CRT expression might contribute to achalasia by similar mechanisms like PLB. For instance, in PLB-knockout mice, cardiac contractility was enhanced, because loss of PLB relieved any inhibition of SERCA function by PLB: Ca^{2+} is more effectively removed from the cytosol and more Ca^{2+} can be released during the systole leading to enhanced force generation in these hearts[36]. A similar mechanism might hold true for CRT in esophagus: lower levels of CRT should also loosen its inhibitory modulation of SERCA function in the esophageal cells and more tension might be generated.

In conclusion, our results provide new insights to understand the mechanisms leading to achalasia. Further studies will be necessary to elucidate the role of impaired Ca^{2+} regulation for development of diseases like achalasia. Some questions which have to be answered are why the Ca^{2+}-regulatory proteins CSQ and CRT are down-regulated, and does down-regulation occur before or after manifestation of the disease.

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CLINICAL RESEARCH

Postprandial transduodenal bolus transport is regulated by complex peristaltic sequence

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Abstract

AIM: To study the relationship between the patterns of postprandial peristalsis and transduodenal bolus transport in healthy subjects.

METHODS: Synchronous recording of chyme transport and peristaltic activity was performed during the fasting state and after administration of a test meal using a special catheter device with cascade configuration of impedance electrodes and solid-state pressure transducers. The catheter was placed into the duodenum, where the first channel was located in the first part of the duodenum and the last channel at the duodenojejunal junction. After identification of previously defined chyme transport patterns the associated peristaltic patterns were analyzed.

RESULTS: The interdigestive phase 3 complex was reliably recorded with both techniques. Of 497 analyzed impedance bolus transport events, 110 (22%) were short-spanned propulsive, 307 (62%) long-spanned propulsive, 70 (14%) complex propulsive, and 10 (2%) retrograde transport. Short-spanned chyme transports were predominantly associated with stationary or propagated contractions propagated over short distance. Long-spanned and complex chyme transports were predominantly associated with propulsive peristaltic patterns, which were frequently complex and comprised multiple contractions. Propagated double wave contraction, propagated contraction with a clustered contraction, and propagated cluster of contractions have been identified to be an integralted part of a peristaltic sequence in human duodenum.

CONCLUSION: Combined impedancometry and manometry improves the analysis of the peristaltic patterns that are associated with postprandial transduodenal chyme transport. Postprandial transduodenal bolus transport is regulated by propulsive peristaltic patterns, which are frequently complex but well organized. This finding should be taken into consideration in the analysis of intestinal motility studies.

Key words: Transduodenal bolus transport; Organization of duodenal peristalsis; Combined impedance manometry

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INTRODUCTION

The spatial and temporal organization of gastrointestinal contraction waves seems to be a more important determinant of the flow of luminal contents than their number and amplitude[1,2]. Therefore, significant advances in understanding intestinal motility disorders could be made by analysis of bolus movement together with peristaltic activity within the gastrointestinal lumen. With manometry alone small intestinal motility patterns can be obtained[3]. However, up to date there are limited observations on the relationship between intestinal chyme transport and peristaltic activity in humans because fluoroscopic studies are limited due to radiation exposure.

Multichannel impedancometry is a newly developed technique to study chyme transport[4]. We performed human studies and demonstrated that multichannel impedancometry is a reliable technique to obtain detailed information about spatial and temporal chyme movements, both in the human esophagus and duodenum[5-9]. Simultaneous impedancometry and pH-monitoring have also been used for characterisation of patterns of gastroesophageal reflux[10,11].

In previous studies we have characterized postprandial duodenal chyme transport patterns[5,6]. Recently, we developed the technique of combined impedancometry and manometry (CIM)[12] and applied it for motility testing in healthy subjects and reflux patients[13-15]. In the present study we used this approach in order to systematically

study the relationship between intestinal chyme transport patterns and peristaltic patterns and to obtain detailed information about peristaltic mechanisms regulating postprandial transduodenal bolus transport.

MATERIALS AND METHODS

Subjects

Ten subjects (7 males and 3 females, mean age 34 years, range 26-36 years) were studied after written informed consent. All healthy volunteers were recruited from the medical staff. They took no medication and had no history of gastrointestinal disease. The study protocol was approved by the local ethical committee of Aachen University.

Methods

Combined impedance and pressure recording: A custom-made combined catheter consisting of 11 impedance segments (each 2 cm long) and 4 semiconductor pressure transducers was used (prototype developed by Dr. Nguyen RWTH-Aachen, Ref. 13). The pressure transducers were located between the impedance channels 1-2, 4-5, 7-8 and 10-11 (intertransducer distance 6 cm) (Figure 1). The cascade configuration of the impedance electrodes allows continuous monitoring of chyme movement and is particularly suitable for analysis of chyme transport patterns[5,6].

Study protocol: After an overnight fast from 10 pm, the catheter was placed transnasaly into the duodenum under fluoroscopic control. All channels were placed in the duodenum. The proximal end of the catheter was located in the first part of the duodenum and the last channel at the duodenojejunal junction (Figure 1). This catheter position yielded information about bolus transport along the whole duodenum. The final position was confirmed by fluoroscopy at the beginning of the studies.

Synchronous recording of chyme transport and peristaltic activity was started after a resting period of at least 20 min following catheter placement. After identification of a phase 3 migrating motor complex, a standard test meal consisting of 500 g of commercially available yogurt with small pieces of fruit (450 kcal, 400 mL, 5.5 g fat, 12.5 g protein, 75 g carbohydrate) was administered, and data were collected for a further 2 h.

Statistical analysis

Impedance and manometry tracings were reconstructed on screen and the patterns were consecutively analyzed. Since we studied transduodenal bolus transport, only impedance signals related to a complete chyme transport over at least 6 cm beginning at the first impedance channel were included and analyzed as previously described as a bolus transport event (BTE)[5,6]. This definition was used to exclude transpyloric movement of gastric contents into the duodenum bulb without initiation of duodenal peristaltsis. BTE were classified according to (a) site of onset (proximal *vs* distal), (b) propulsion direction (propulsive *vs* retropulsive), (c) propagation distance (short-spanned $\leqslant$ 8 channels or $\leqslant$ 16 cm *vs* long-spanned > 8 channels or > 16 cm), (d) number of components (simple = one component *vs* complex = multiple components). Thus the transport patterns were: (a) short-spanned propulsive, (b) retrograde, (c) long-spanned propulsive, and (d) complex propulsive[5,6,8]. Of note, our previous validation study[6] demonstrated that long-spanned BTE are associated with a significant drop of intraluminal pH and change of electrical conductivity, thus indicating real chyme movement originating from the stomach.

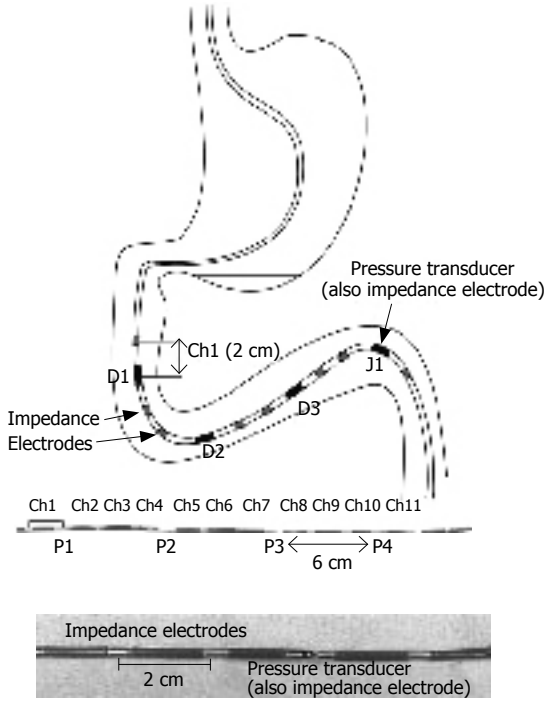

Figure 1 Modified manometry catheter for concurrent impedance-manometry procedure. The 4 semiconductor solid-state pressure transducers (P1-P4) serve also as impedance electrodes and are placed at 6 cm distance each. The 8 impedance electrodes (4 mm length) are arranged between the pressure transducers at a distance of 16 mm. Together with the pressure transducers, they form 11 impedance segments, each 2 cm long (Ch1-Ch11). The solid-state pressure transducers are located exactly between the impedance channels 1-2, 4-5, 7-8, and 10-11, respectively. The first channels were located at the first part of the duodenum.

After identification and classification of the chyme transport patterns the corresponding peristaltic sequences were analyzed. Firstly, the peristaltic nature of the associated contractions was characterized as (a) stationary (isolated contraction observed in only one channel) or (b) propagated (contraction detected over 2, 3 or 4 pressure channels = 6, 12 and 18 cm). Secondly, propagated contractions were classified according to Summers *et al*[16] to be: (a) propagated contraction with single wave contraction (1 contraction), (b) propagated contraction with a double wave contraction (2 contractions) or propagated contraction with a clustered contraction (> 2 contractions occurring at a rate of 5 s) (c) propagated cluster of contractions (clustered contractions occurring at more than one pressure channel) as shown in Figure 2. A double spike wave was considered to be single wave. Contractions that were observed between the BTEs were not included for analysis. Data are expressed as total number of events counted.

RESULTS

Combined impedance-manometry during the interdigestive state

During the interdigestive phase 2 there were irregular motility activities as recorded by manometry and irregular chyme transport events as recorded by impedance-manometry (Figure 3, upper panel). Both impedance and manometry recorded the same features of the phase 3 complex (Figure 3, lower panel).

Combined impedance-manometry during the postprandial state

The postprandial manometry tracings showed irregular peristaltic activity, which is difficult to analyse (Figure 4, upper panel). However, with combined impedance and manometry the relationship between transduodenal bolus transport event and associated peristaltic activity could be investigated in more detail (Figure 4, middle panel). Plotting the tracings at high resolution, the patterns of chyme transport as recorded by impedance and of peristalsis as recorded by manometry were analysed systematically (Figure 4, lower panel).

Impedance bolus transport patterns

A total number of 564 BTEs were counted. Sixty seven BTEs (12%) were excluded, because they could not be clearly classified according to the impedance criteria. Of the remaining 497 BTEs the distribution of the impedance patterns was: (a) short-spanned propulsive transport, 110 events (22%), long-spanned propulsive transport, 307 events (62%), and (d) complex transport, 70 events (14%) and retrograde transport events, 10 (2%).

Relationships between impedance transport patterns and manometry peristaltic patterns

The relationship between impedance transport patterns and manometry peristaltic patterns could be classified as followed (Figure 5): (a) of the short-spanned propulsive chyme transports (110 events) the majority of them was associated either with a stationary contraction (43 events or 39%) or a single contraction propagated over only 2 pressure channels (64 events or 58%); (b) all long-spanned propulsive chyme transports (307 events) were associated with a propagated contractions; (c) all complex chyme transport patterns (70 events) were associated with propagated contractions, particularly over 4 pressure channels; (d) retrograde chyme transport was rare (10 events) and are associated either with a stationary contraction (7 events) or retrograde propagated single wave contraction (3 events).

Of the long-spanned bolus transport events (307 events), 92 BTEs (30% of them) were associated with a propagated contraction over 2 or 3 pressure channels (6 -12 cm), and 215 BTEs (70% of them) were associated with a propagated contraction over 4 pressure channels (18 cm).

Of the long-spanned propulsive chyme transports over 4 channels (215 events), 61 events (28% of them) were associated with a propagated single wave contraction, and 154 events (72% of them) were associated with a propagated double wave contraction or a propagated contraction with a clustered contraction. None of these bolus transport was associated with a propagated cluster of contractions.

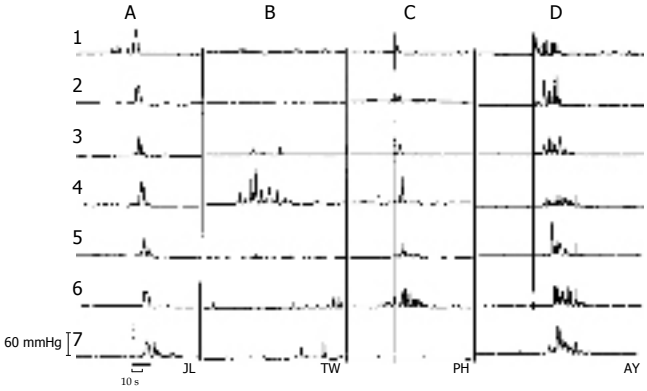

Figure 2 Classification of the peristaltic patterns according to Summers *et al*[16]. The peristaltic pattern can be classified to be (A) propagated single (double spike) wave contraction, (B) isolated (stationary) cluster of contractions, (C) propagated contraction with a clustered contraction, and (D) propagated cluster of contractions.

All complex propulsive chyme transports (70 events) were associated with propagated contractions over 4 channels (18 cm): 48 events (68% of them) were associated with a propagated double wave contraction or a propagated contraction with a clustered contraction, and 22 events (32% of them) were associated with a propagated cluster of contractions. None of these bolus transport was associated with a propagated single wave contraction.

Examples of the bolus transport events and their associated peristaltic patterns are shown in Figures 6-8.

DISCUSSION

Only few studies in man have directly analysed the spatial and temporal relationship between the patterns of chyme flow and patterns of peristaltic contraction waves in the duodenum, particularly during the postprandial state and in details. This study was performed to directly address this issue using the newly developed and validated technology of combined impedance and manometry for motility testing[12-15]. The daisy-chained configuration of the impedance electrodes in the present system differ significantly from other systems[17-19], where the impedance electrode pairs are located far from each other. As shown in previous impedance studies[5,6] this catheter configuration offers a high spatial resolution for detailed monitoring of bolus transport patterns. The incorporated solid state pressure transducers allow the concurrent analysis of the corresponding contractile events.

As shown in Figure 3, the phase 3 complex is recorded identically by both techniques, showing the well-known characteristics of the migrating motor complex, similar to recent findings by Imam *et al*[17]. During the postprandial phase, chyme transport and associated peristaltic activity can be accurately monitored, and thus a large number of bolus transport event (BTE) was obtained for detailed analysis.

Considering the manometry tracings alone, it has been

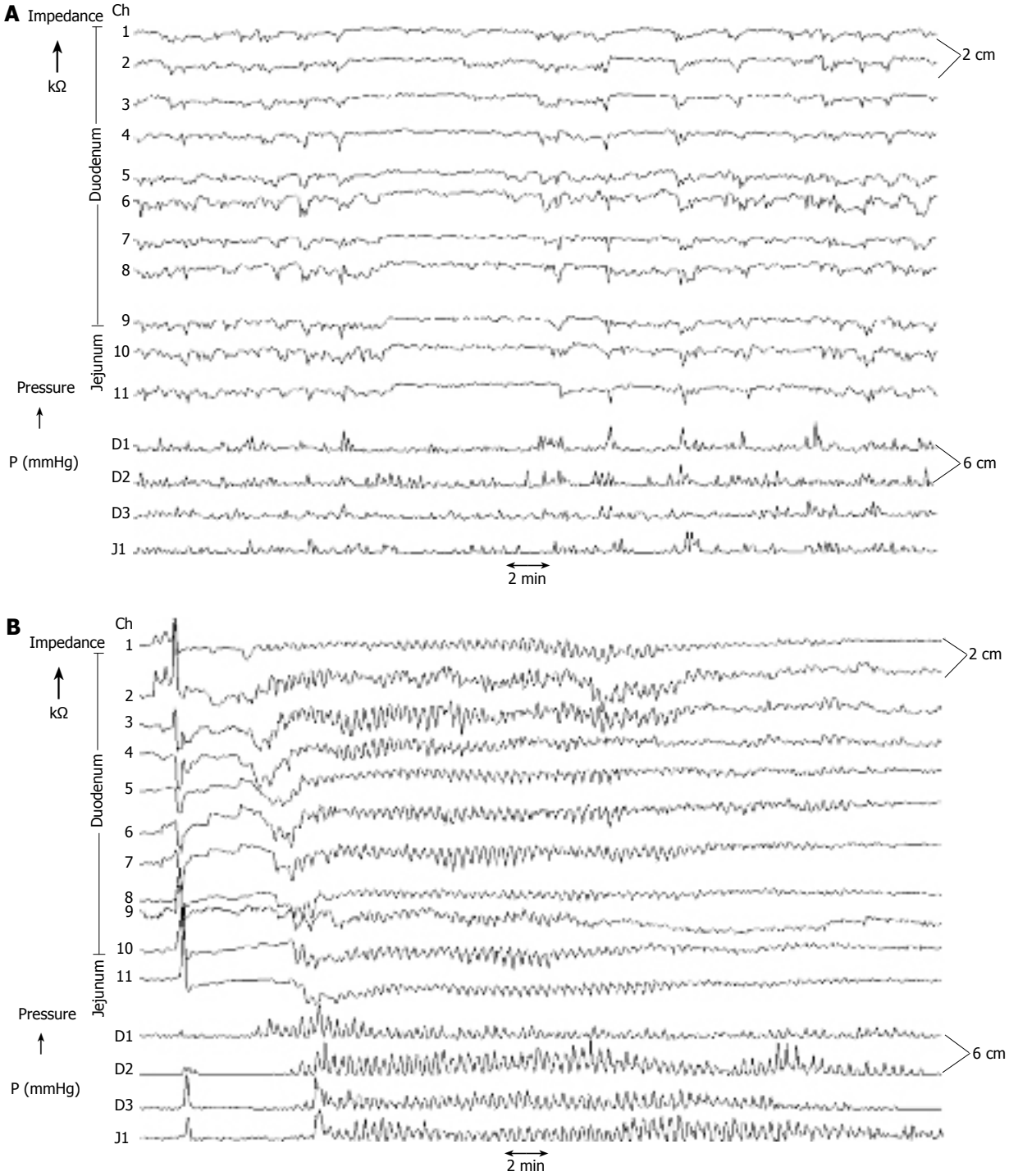

Figure 3 Concurrent Impedance Manometry (CIM) tracings. Upper panel: During the interdigestive phase irregular chyme transport at the impedance channels and irregular motor activities at the pressure channels are observed. Lower panel: A phase 3 complex displays nearly identical features with frequent changes of both pressure and impedance.

shown to be difficult to characterize contraction waves to be stationary or propagated[20]. As shown in Figure 4 this problem can be overcome by the combined technique as shown in the recent study. After a bolus transport event (BTE) had been identified with impedance in the present study, the organization of the associated contraction waves could be analyzed. We did not include contraction waves that occurred between the bolus transport events, as compared to previous studies[1,3,20], which analyzed the overall motility activity. Therefore, the results are not comparable. As only impedance events representing a bolus transport over at least 6 cm were analysed, the present results provide data about the postprandial organization of peristaltic activity in association with a transduodenal bolus transport. Transpyloric chyme movement was not included in the present studies.

A major finding of the present study is the close relationship between transduodenal bolus transport patterns and peristaltic contraction patterns. The results showed that long-spanned chyme transport patterns are predominantly associated with propagated peristaltic patterns, whereas short-spanned chyme transport patterns

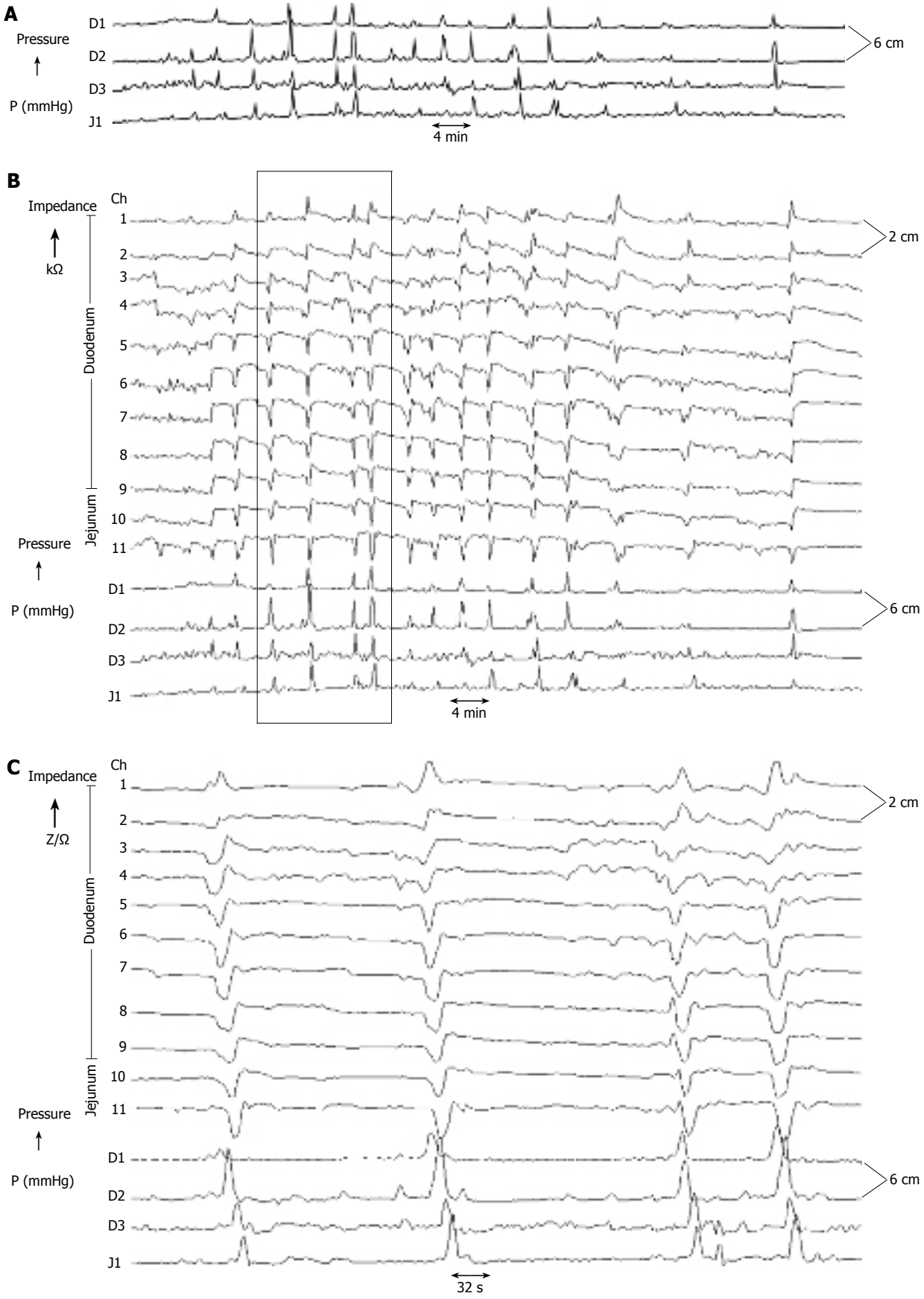

Figure 4 Concurrent Impedance Manometry (CIM) tracings after a test meal. Upper panel: Low time scaled manometry tracings of the postprandial state. Middle panel: Low time scaled impedance manometry tracings of the same period as above showing several bolus transport events with associated peristaltic activities. Lower panel: High time scaled impedance manometry tracings of the box allowing identification and classification of bolus transport patterns as well as analysis of associated peristaltic patterns.

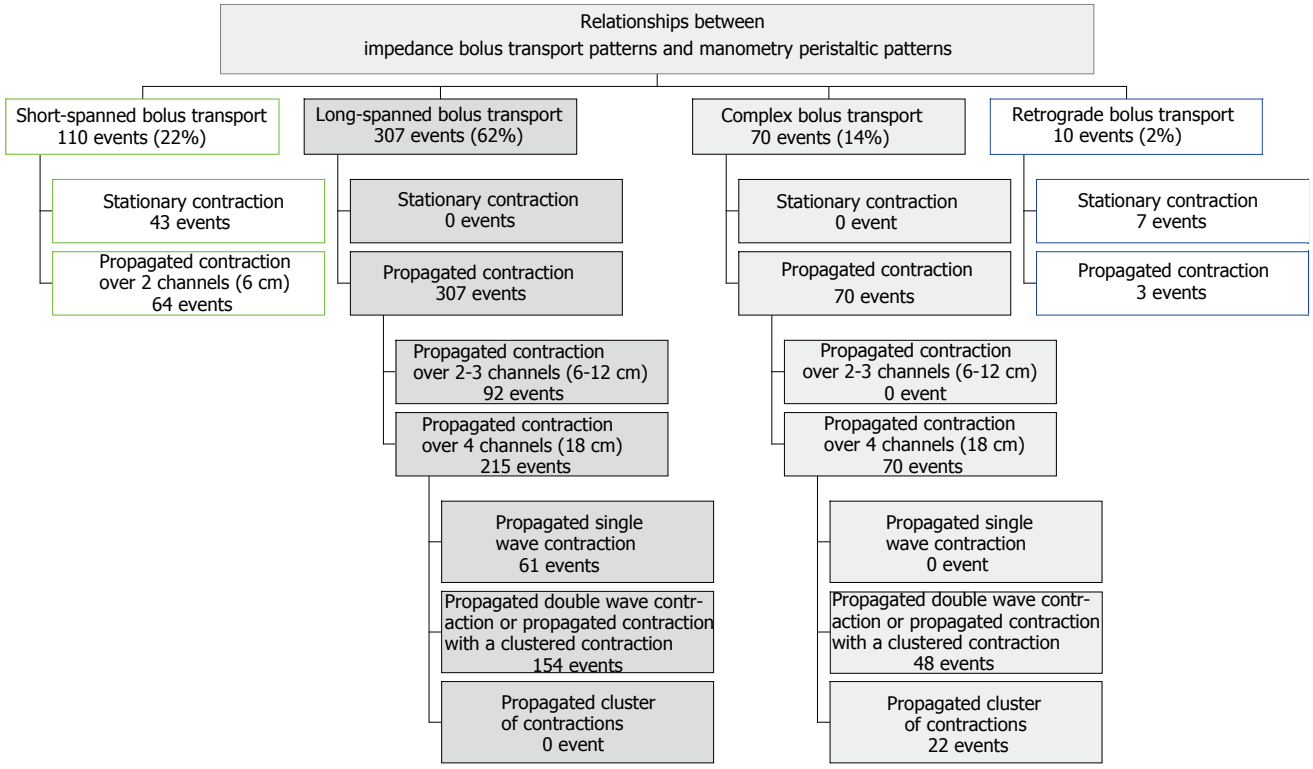

Figure 5 Distribution of bolus transport patterns and associated peristaltic patterns (see text for details).

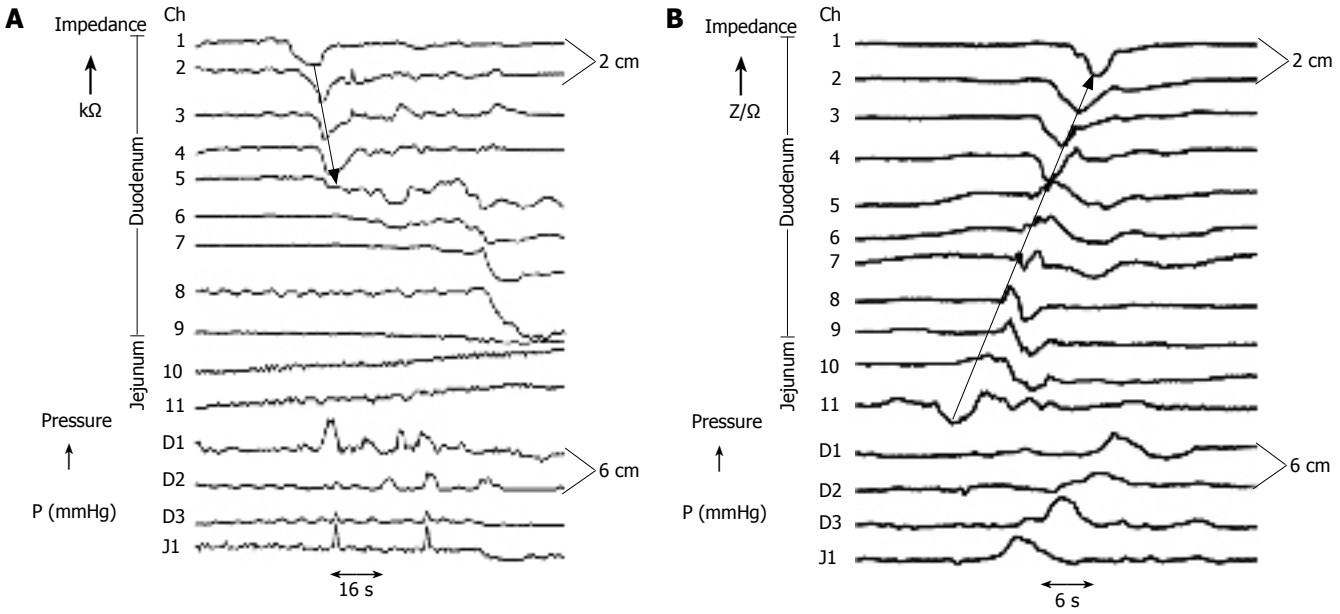

Figure 6 Examples of simple bolus transports and associated peristalsis. Upper panel: Short-spanned bolus transport with associated stationary contraction. Lower panel: Retrograde bolus transport with associated retrograde peristalsis.

are frequently related to stationary contractions or propagated contractions over short distance (6 cm). The data are consistent with results of a recent study[17] showing that impedance corresponds better with fluoroscopic flows than manometry and that recording of pressure events can underestimate even flow events of substantial length. However, the quantitative data of this study are not comparable to ours because there are important differences with respect to study design and analysis algorithm: (a) in this study the impedance segments were spaced 5 cm apart from each other as compared to the cascade configuration in our study, thus, the recording of impedance patterns differs substantially; (b) all impedance events with a drop of 12% or more below baseline, even if detected in only one impedance segment (spread distance < 5 cm), were included as compared to impedance signals of a complete bolus passage over 6 cm in our study. Since an impedance drop only indicates arrival of a bolus front but not always a complete bolus passage, it might represent transpyloric chyme movement into the proximal duodenum, which is

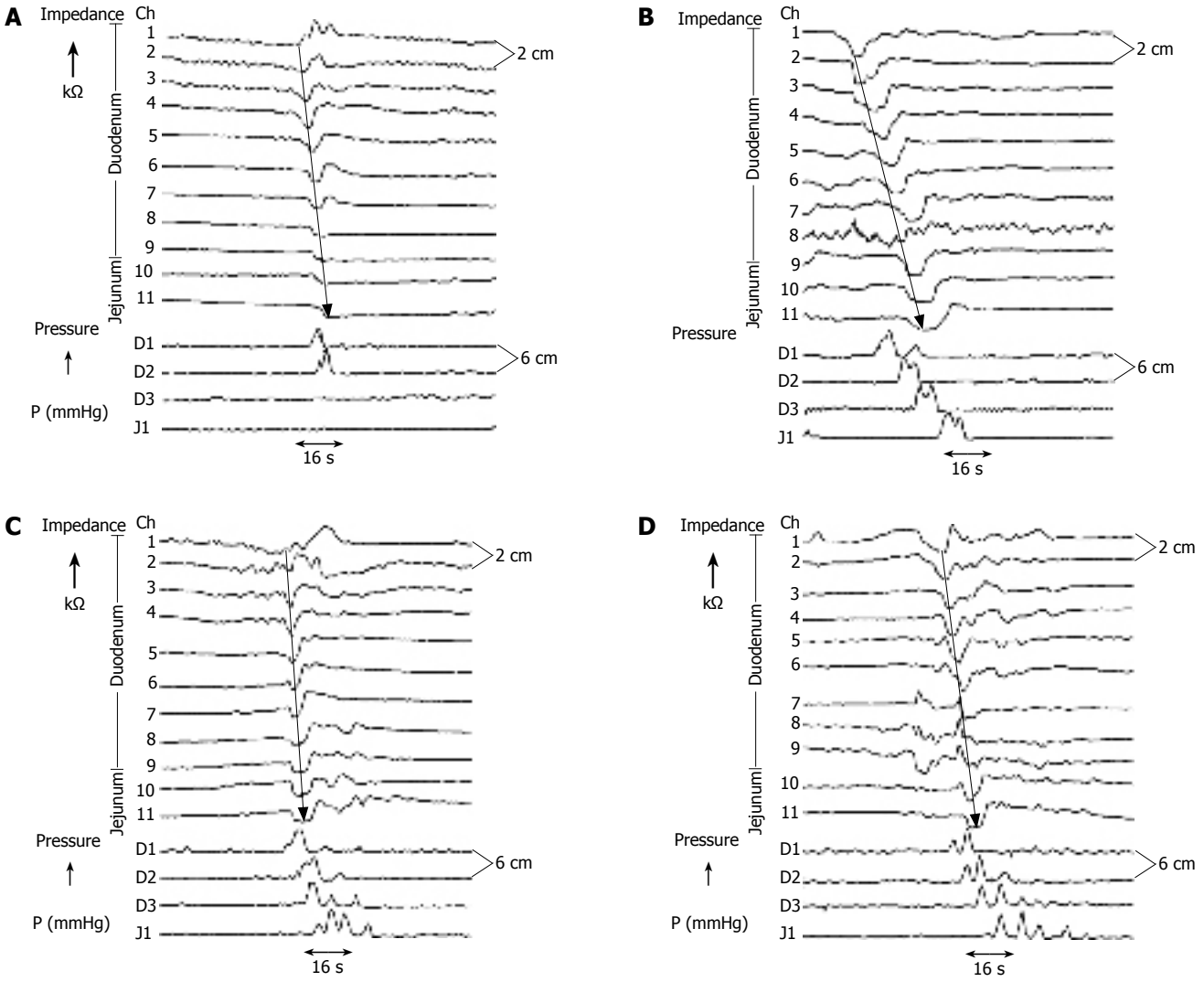

Figure 7 Examples of long-spanned bolus transports and associated peristalsis. (**A**) A long-spanned bolus transport with the bolus front traversing the whole duodenum. The associated peristaltic sequence displayed a propagated single wave contraction from D1 to D2. This peristaltic pattern was classified to be simple (propagated single wave contraction). (**B**) A long-spanned bolus transport traversing the whole duodenum. The associated peristaltic sequence displayed a propagated contraction with a double wave contraction at channel D1 and double spike contractions at D2, D3 and J1. This peristaltic pattern was classified to be complex (propagated contraction with a double wave contraction). (**C**) A long-spanned bolus transport with the bolus front traversing the whole duodenum. The associated peristaltic sequence displayed single wave contractions at channel D1-D3 and a clustered contractions at J1. This peristaltic pattern was classified to be complex (propagated contraction with a clustered contraction). (**D**) A long-spanned bolus transport with the bolus front traversing the whole duodenum. The associated peristaltic sequence displayed double wave contractions at channel D1 and a clustered contractions at D2, D3 and J1. This peristaltic pattern was classified to be complex (propagated cluster of contractions).

detected at the first duodenal channel as compared to a transduodenal bolus transport over at least 6 cm.

Another major finding is related to the motor mechanisms regulating transduodenal bolus transport. Distinct peristaltic patterns have been identified. Our data showed, that if a bolus is propelled over a long distance (> 4 pressure channels or 18 cm), it is frequently associated with a complex peristaltic sequence, which can be either a propagated contraction with double wave contraction, a propagated contraction with a clustered contraction, or rarely, a propagated cluster of contractions. The finding that cluster of contractions (double wave contraction, clustered contraction) are an integral part of a peristaltic sequence associated with a transduodenal bolus transport is new. The retrospective analysis of these contraction patterns without the associated impedance tracings frequently fails to clearly identify the clustered contractions to be a part of bolus-associated peristaltic sequence. These results may explain the facts that the analysis and classification of all contraction waves in human small intestine is difficult, and most of them have been classified to be stationary[20].

The dominant patterns of propulsive bolus transports with associated propulsive peristaltic patterns support the existence of a precise spatial and temporal organization of the contraction waves in human duodenum during the postprandial state[21-24]. Two recent studies investigating the relationships between antral contraction, transpyloric fluid flow and duodenal motility observed that transpyloric fluid transport is associated with duodenal propagation[18,21]. The results of the present study strongly support previous reports showing that coordinated duodenal contraction waves are an important determinant regulating antroduodenal chyme flow[22,24,25], as well as gastric emptying[26].

The recent finding of different propulsive bolus transport patterns associated with different propulsive

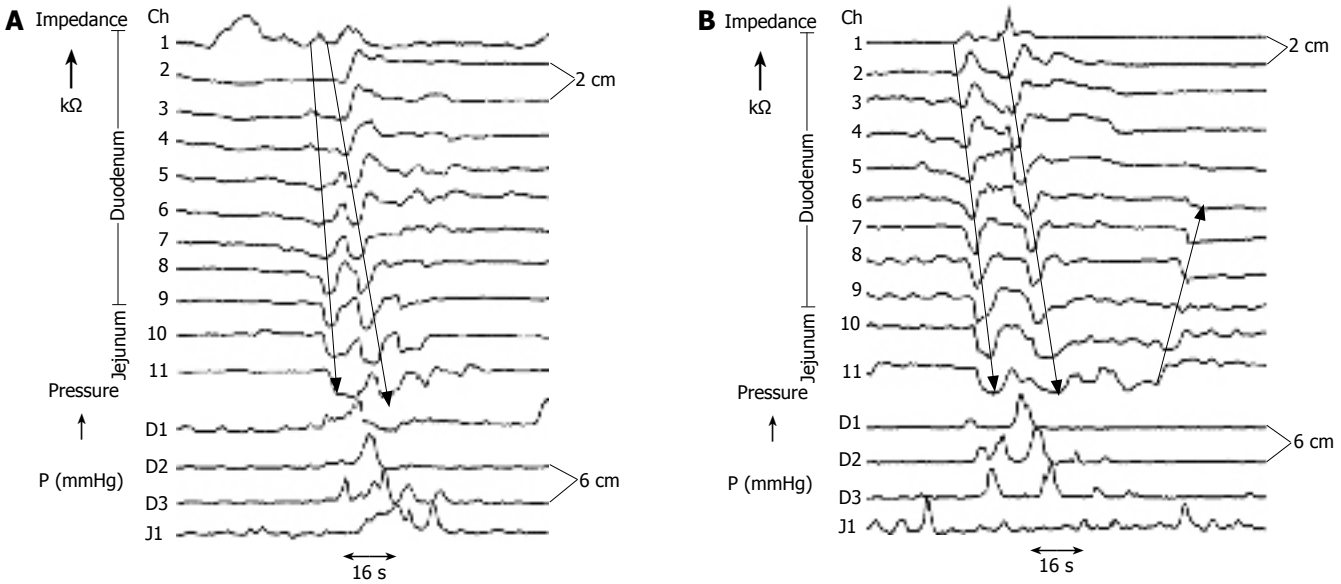

Figure 8 Examples of complex bolus transports and associated complex peristalsis. (**A**) A complex bolus transport with two components propelling with different propulsion velocity as illustrating by the arrows. The associated peristaltic sequence is complex showing a long lasting double spike contraction at D1, a single wave contraction at D2, a clustered contraction at D3 and a multispike double wave contraction at J1. (**B**) A complex bolus transport comprising two boluses following each other very rapidly as illustrating by the anterograde arrows. The associated peristalsis showed two separate peristaltic sequences which seemed to be connected together by a interpolate contraction wave at D2. A retrograde bolus movement (retrograde arrow) seemed to be derive from a retrograde peristaltic sequence with a single contraction wave seen at J1.

peristaltic patterns underlines the physiological difference between the duodenum and the esophagus, where the bolus transport patterns are highly uniform and the peristaltic sequences are predominantly simple[7,13]. This finding support the view that the duodenum is not only a conduit but also an active segment, which is able to generate contractions transporting and mixing gastric contents together with duodenal juices into the jejunum[27-31]. Since previous studies[32,33] indicated that contraction patterns in the duodenum are quite different from those in the jejunum, further studies should examine, if similar bolus transport patterns and peristaltic patterns will be found in other segments of the gut.

The answers about the question regarding how complex peristaltic sequences can be regenerated during a bolus transport can be sought in results of electrophysiological studies. Intestinal motility is considered to be controlled by interaction between myogenic, neural and humoral factors[34-35]. In recent studies several motility patterns have been characterized as peristaltic or pendular, stationary or propagating, or twitch or segmental[36-37]. Furthermore, several electrical signals have been shown to be associated with different types of contractions including slow waves, spikes, or bursts[36-38]. Therefore, peristalsis with associated propagated peristaltic waves should not be regarded as a simplex reflex, bur rather as a co-ordinated locomotor pattern, which can be induced either by fluid distension, local stretch, or mucosal stroking[36]. Careful examination of the spatial and temporal relationship between spontaneous slow waves and peristaltic waves showed that they seem to constitute two separate electrical events that may drive two different mechanisms of contractions[39]. Slow waves are not in rhythm with peristaltic waves and they may occur in different groupings and patterns. These waves may travel in the same or in opposite directions from each other and may propagate in the oral or caudal direction, and therefore, may modulate each other. Similarly, slow waves and spikes seem to be propagated by different mechanisms through different cell networks[32]. Thus, the spatial and temporal characteristics of contraction in the small intestine seem to be determined not only by the direction of the slow wave but also whether or not spikes are generated after these slow waves[32,40].

There are 2 limitations of the recent studies, which should be evaluated in further studies: (a) since the composition of the test meals significantly affect small intestine motility, it remains to be determined, if the recent bolus transport and peristaltic patterns will be the same by using different test meals; (b) since the intertransducer distance significantly affect the recognition of propagated pressure waves, it remains to be determined, if more closely spaced recording points with 1-2 cm apart may provide more accurate data.

In summary, combined impedancometry and manometry in human duodenum provides detailed data about the relationship between the organization of contraction waves and the patterns of chyme flow during the postprandial state. This technique improves the analysis of intestinal motility. Several postprandial peristaltic patterns associated with transduodenal bolus transport have been identified showing that cluster of contractions constitutes an integral part of the peristaltic sequence associated with transduodenal bolus transport. The results provide new insights into the peristaltic mechanisms that are associated with transduodenal chyme transport and maintain the physiological function of the duodenum. The present results clearly indicate that comprehensive motility testing in the small intestine, particularly during the postprandial state, should be performed using the combined technique. The present data will serve as basis

findings forwarding clarifying small intestinal motor dysfunction.

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CLINICAL RESEARCH

Comparative clinical trial of S-pantoprazole *versus* racemic pantoprazole in the treatment of gastro-esophageal reflux disease

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Abstract

AIM: To compare the efficacy and tolerability of S-pantoprazole (20 mg once a day) versus racemic Pantoprazole (40 mg once a day) in the treatment of gastro-esophageal reflux disease (GERD).

METHODS: This multi-centre, randomized, double-blind clinical trial consisted of 369 patients of either sex suffering from GERD. Patients were randomly assigned to receive either one tablet (20 mg) of S-pantoprazole once a day (test group) or 40 mg racemic pantoprazole once a day (reference group) for 28 d. Patients were evaluated for reduction in baseline on d 0, GERD symptom score on d 14 and 28, occurrence of any adverse effect during the course of therapy. Gastrointestinal (GI) endoscopy was performed in 54 patients enrolled at one of the study centers at baseline and on d 28.

RESULTS: Significant reduction in the scores (mean and median) for heart burn ($P < 0.0001$), acid regurgitation ($P < 0.0001$), bloating ($P < 0.0001$), nausea ($P < 0.0001$) and dysphagia ($P < 0.001$) was achieved in both groups on d 14 with further reduction on continuing the therapy till 28 d. There was a statistically significant difference in the proportion of patients showing improvement in acid regurgitation and bloating on d 14 and 28 ($P = 0.004$ for acid regurgitation; $P = 0.03$ for bloating) and heart burn on d 28 ($P = 0.01$) between the two groups, with a higher proportion in the test group than in the reference group. Absolute risk reductions for heartburn/acid regurgitation/bloating were approximately 15% on d 14 and 10% on d 28. The relative risk reductions were 26%-33% on d 14 and 15% on d 28. GI endoscopy showed no significant difference in healing of esophagitis ($P = 1$) and gastric erosions ($P = 0.27$) between the two groups. None of the patients in either group reported any adverse effect during the course of therapy.

CONCLUSION: In GERD, S-pantoprazole (20 mg) is more effective than racemic pantoprazole (40 mg) in improving symptoms of heartburn, acid regurgitation, bloating and equally effective in healing esophagitis and gastric erosions. The relative risk reduction is 15%-33%. Both drugs are safe and well tolerated.

Key words: Gastro-esophageal reflux disease; Pantoprazole; Efficacy; Tolerability

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INTRODUCTION

The primary treatment goals in patients with gastroesophageal reflux disease (GERD) are relief of symptoms, prevention of symptom relapse, healing of erosive esophagitis, and prevention of complications of esophagitis[1]. Proton pump inhibitors (PPIs) block the final step in acid production and hence are the most effective inhibitors of acid secretion[2]. Recent advances in analytical methods for the separation of enantiomers of PPIs have led to a considerable interest in the stereoselective pharmacokinetics of PPI enantiomers[3].

Pantoprazole, a selective and long acting PPI, is a chiral sulfoxide that is used clinically as a racemic mixture of S-pantoprazole and R (+) pantoprazole. The pharmacokinetics of R and S isomers of pantoprazole vary widely in extensive and poor metabolizers[4]. The use of a single isomer avoids this variation and offers predictable pharmacokinetics. Animal studies have shown that S-pantoprazole is more potent (1.5 to 1.9 times) and effective (3 to 4 times) than racemate in inhibiting gastric lesions in different pre-clinical models[3,5], suggesting that in human patients 20 mg of S-pantoprazole would be at least equivalent in efficacy to 40 mg of racemic pantoprazole. The present study was to compare the efficacy and tolerability of S-pantoprazole (20 mg) *versus* racemic pantoprazole (40 mg) in the treatment of gastro-oesophageal reflux disease (GERD) in adult patients.

MATERIALS AND METHODS

Materials

S-pantoprazole was synthesized by Emcure Pharmaceuticals Ltd. Tablets of S-pantoprazole (20 mg) and racemic pantoprazole (40 mg) were provided by the same source in coated opaque packets containing 28 tablets each without labeling the identity of the contents.

Methods

This multi-centre, randomized, double blind comparative study permitted by Drugs Controller General of India (DCGI) was conducted in compliance with the 'Guidelines for Clinical Trials on Pharmaceutical Products in India-GCP Guidelines' issued by the Central Drugs Standard Control Organization, Ministry of Health, Government of India (http://www.cdsco.nic.in/html/GCP1.html). The Ethical Committee approval was taken from Sharada Clinic Ethical Committee, Karad for Dr. S Erram, Independent Ethical Committee for Dr. H Thacker, Independent Ethical Committee of Surya Hospital, Pune for Dr. V Pai, Dr. V Mandora and Dr. J Shinde. Written informed consent was obtained from the subjects. The study was initiated on November 7, 2004 and completed on February 25, 2005.

Subjects

Patients of either sex, aged 18-65 years, with clinically confirmed GERD, were enrolled in the study after providing written informed consent. Patients with known hypersensitivity to pantoprazole and any major hematologic, hepatic, metabolic, gastrointestinal or endocrine disorder requiring any other anti-GERD medication and women who were pregnant or lactating or child bearing potential and not practicing effective method of contraception were excluded from the study.

Patients were randomized in blocks of ten, as per the computer generated randomization chart (www.randomization.com) into two treatment groups: one group receiving 20 mg S-pantoprazole once daily for 28 d (test group) while the other group receiving 40 mg racemic pantoprazole once daily (reference group) for the same period. The identity of the treatment allocated was unknown to either investigators or patients. The reference and test medications had a similar appearance, shape and size and could not be distinguished from each other based on their external appearance. All the patients completed the 28 d therapy during which they were followed up twice on d 14 and 28. Medication compliance was checked by counting the number of tablets left in the packet, if any.

Scoring of symptoms

Heartburn, regurgitation and dysphagia are symptoms of GERD. Although nausea and bloating are symptoms of dyspepsia which can be an overlapping condition in patients with GERD, this study included patients with symptoms of nausea and bloating also in order to capture the overall clinical benefit. The severity of heartburn, acid regurgitation, bloating and nausea was scored as follows: 0 = none, no symptom; 1 = mild, occasional symptoms that did not affect normal activities; 2 = moderate, frequent symptoms or symptoms that affected normal activities; 3 = severe, constant symptoms. The severity of dysphagia was scored as follows: 0 = normal; 1 = occasional sticking of solids; 2 = swallowing semisolids and Pureed food; 3 = swallowing liquids only. Scores were recorded for all the patients at the initiation of the study and then on d 14 and 28. Improvement in symptoms was defined as reduction in baseline symptom score. Assessment of within-group efficacy was done by comparing symptom scores on d 14 and 28 with baseline value on d 0 of the study. Assessment of between-group efficacy was done by comparing the proportions of patients showing improvement in symptoms in each group. GI endoscopy was done in 54 patients enrolled at one of the study centers on d 0 and 28. Tolerability profile was assessed by comparing the incidence of possible drug-related adverse effects in each group on d 14 and 28 of the study.

Table 1 Baseline demographic variables

Variable	Reference group	Test group
n	182	187
Male: Female	115:67	114:73
Mean age ± SD (yr)	42.3 ± 11.7	42 ±12.3

Statistical analysis

The results were analyzed using Fisher's exact test (two sided) for difference in proportions, Friedman's test (non-parametric repeated measures ANOVA) for comparison of between-days score (variation amongst column medians). For this, $P < 0.05$ was considered statistically significant. Individual differences between columns (d 0 *vs* d 14 and d 0 *vs* d 28) were assessed by Dunn's multiple comparisons test. The GraphPad InStat (version 3.06, 32 bit for Windows, September 11, 2003, GraphPad Software, San Diego California USA, www.graphpad.com) and WINPEPI suite (Abramson JH (2004) WINPEPI (PEPI-for-Windows) computer programs for epidemiologists as well as Epidemiologic Perspectives & Innovations (2004, 1:6) softwares were used for statistical analysis. UBC clinical significance calculator (from http://www.healthcare.ubc.ca/calc/clinsig.html) was used to calculate the absolute risk reduction (ARR), relative risk reduction (RRR) and number needed to treat (NNT). ARR is the absolute arithmetic difference in rates of bad outcomes between experimental and control participants in a trial, calculated as experimental event rate minus control event rate. RRR is the proportional reduction in rates of bad outcomes between experimental and control participants in a trial, calculated as experimental event rate minus control event rate divided by control event rate. NNT is the number of patients who need to be treated to achieve one additional favorable outcome, calculated as 1/ARR.

RESULTS

Three hundred and sixty-nine patients (229 males and 140 females) with symptoms of GERD were enrolled in the study after providing written informed consent. Baseline demographic variables of the two groups are shown in Table 1. There was no statistically significant difference between the two groups at baseline.

Table 2 Efficacy in the reference (racemic pantoprazole 40 mg, *n* = 182) and test (S-pantoprazole 20 mg, *n* = 187) groups

Symptoms		d 0		d 14		d 28		*P*[3]
		Ref	Test	Ref	Test	Ref	Test	
Heart burn	Mean ± SD	1.8 ± 0.8	1.9 ± 0.8	1.2 ± 0.8	1.2 ± 0.9	0.6 ± 0.9	0.6 ± 0.8	< 0.0001
	Median (Percentile 25th, 75th)	2 (1.2)	2 (1.3)	1 (1.2)[2]	1 (0.5.2)[1]	0 (0.1)[2]	0 (0.1)[1]	
Regurgitation	Mean ± SD	1.6 ± 0.9	1.7 ± 0.9	1.1 ± 0.8	1 ± 0.8	0.5 ± 0.8	0.4 ± 0.6	< 0.0001
	Median (Percentile 25th, 75th)	2 (1.2)	2 (1.2)	1 (0.2)[2]	1 (0.2)[1]	0 (0.1)[2]	0 (0.1)[1]	
Bloating	Mean ± SD	1.4 ± 0.9	1.4 ± 1	0.9 ± 0.9	0.8 ± 0.8	0.5 ± 0.8	0.3 ± 0.6	< 0.0001
	Median (Percentile 25th, 75th)	1 (1.2)	2 (1.2)	1 (0.2)[2]	1 (1.2)[1]	0 (0.1)[2]	1 (1.1)[1]	
Nausea	Mean ± SD	1.4 ± 1	1.3 ± 1	0.8 ± 0.9	0.7 ± 0.8	0.4 ± 0.8	0.4 ± 0.7	< 0.0001
	Median (Percentile 25th, 75th)	2 (0.2)	1 (0.2)	1 (0.2)[2]	0 (0.1)[1]	0 (0.0.25)[2]	0 (0.1)[1]	
Dysphagia	Mean ± SD	0.3 ± 0.6	0.5 ± 0.8	0.2 ± 0.6	0.2 ± 0.5	0.1 ± 0.4	0.1 ± 0.4	< 0.001
	Median (Percentile 25th, 75th)	0 (0.0)	0 (0.1)	0 (0.0)	0 (0.0)[1]	0 (0.0)	0 (0.0)[1]	

[1,2] Dunn's multiple comparisons test; [3]*P* value for ANOVA (Friedman's test).

Table 3 Between-group efficacy in improvement of symptoms

Decrease compared to baseline (d 0) in score	Patients showing improvement in symptoms (*n*)											
	Heart burn				Regurgitation				Bloating			
	On d 14		On d 28		On d 14		On d 28		On d 14		On d 28	
	Ref	Test	Ref	Test	Ref	Test	Ref	Test	Ref	Test	Ref	Test
Decrease by 1	89	93	56	71	90	111	76	86	62	81	69	65
Decrease by 2	9	21	61	67	6	12	52	52	11	14	36	38
Decrease by 3	0	1	11	15	0	2	8	20	3	2	9	21
Patients who improved (*n*)	98	115	128	153	96	125	136	158	76	97	114	124
Patients with symptoms (*n*)	172	179	172	179	165	170	165	170	149	143	149	143
Fisher's test, *P*	0.19		0.01		0.004		0.004		0.004		0.03	

No significant differences were present in the baseline symptom scores in both groups. In both treatment groups, significant reductions in the mean and median scores for heart burn ($P < 0.0001$), acid regurgitation ($P < 0.0001$), bloating ($P < 0.0001$), nausea ($P < 0.0001$) and dysphagia ($P < 0.001$) were achieved on d 14 with further reduction on continuing the therapy till d 28 (Table 2). The percentage of patients in the reference group, achieving improvement in heart burn, acid regurgitation, bloating, nausea and dysphagia was 57.0%, 58.2%, 51.0%, 66.4% and 58.6% respectively at the end of 14 d therapy, and 74.4%, 82.4%, 76.5%, 81.3% and 79.3% respectively at the end of 28 d therapy. The percentage of patients in the test group, achieving improvement in heart burn, acid regurgitation, bloating, nausea and dysphagia was 64.3%, 73.5%, 67.8%, 72.7% and 67.8% respectively at the end of 14 d of therapy, and 85.5%, 92.9%, 86.7%, 81.3% and 84.7% respectively at the end of 28 d of therapy. There was a statistically significant difference in the proportion of patients showing improvement in acid regurgitation and bloating on d 14 and 28 ($P = 0.004$ for acid regurgitation; $P = 0.03$ for bloating), and heart burn on d 28 ($P = 0.01$) between the two groups, with a higher proportion in the test group than in the reference group (Table 3, Figure 1).

Of the 54 patients who underwent GI endoscopy, baseline findings of esophagitis and erosions were present in 17 (68%) and 6 (2.4%) patients out of 25 patients in the reference group and 21 (72.4%) and 2 (0.7%) patients out of 29 patients in the test group respectively. Twenty-eight days after therapy, esophagitis and erosions were present in 7 (28%) and 6 (2.4%) patients out of 25 patients in the reference group and 9 (31%) and 3 (1.03%) patients out of 29 patients in the test group respectively. There was no significant difference in both treatment groups in healing of esophagitis ($P = 1$) and erosions ($P = 0.27$) (Table 5).

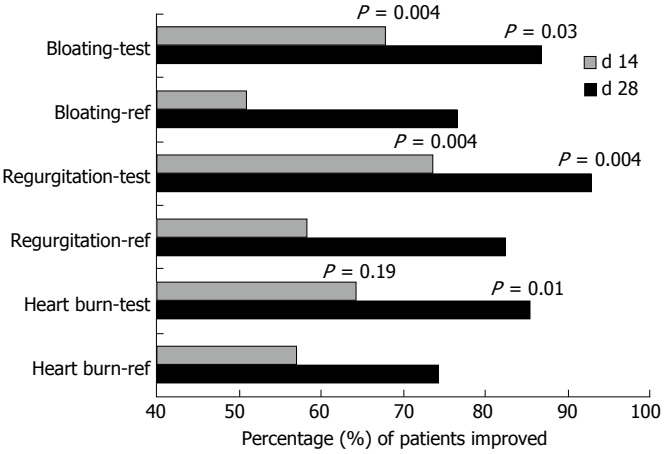

Figure 1 Percentage of patients showing improvement in heartburn, acid regurgitation and bloating in two treatment groups on d 14 and 28.

None of the patients in either group reported any adverse effect during the course of the study. Both drugs were well tolerated.

DISCUSSION

All PPIs are chiral compounds. Chirality can introduce marked selectivity and specificity into the way the drug is handled by the body and how the compound interacts

Table 4 Absolute risk reduction (95% CI for differences between proportions), relative risk reduction (RRR) and number needed to treat (NNT)

Symptoms, d	Absolute risk reduction[1] (95% CI for difference in improvement) (%)	RRR (%)	NNT
Heartburn, d 28	+ 11.1 (2.7-19.4)	15	9
Acid regurgitation, d 14	+ 15.3 (5.3-25.0)	26	7
Acid regurgitation, d 28	+ 10.5 (3.6-17.5)	13	10
Bloating, d 14	+ 16.8 (5.7-27.9)	33	6
Bloating, d 28	+ 10.2 (1.4-19.0)	13	10

[1]Significant differences in favor of test group were analyzed.

with the receptor or enzyme binding sites in some cases. Overall, this may lead to variations in pharmacokinetic and pharmacodynamic properties and differences in safety and toxicity profiles[6].

The present study showed that symptoms of GERD *viz* heart burn, acid regurgitation, bloating, nausea and dysphagia improved significantly in both treatment groups. The results also showed that a significantly higher proportion of patients treated with 20 mg S-pantoprazole achieved improvement in heart burn, acid regurgitation and bloating as compared to patients treated with 40 mg racemic pantoprazole (Table 3, Figure 1). The absolute risk reduction was approximately 15% on d 14 and 10% on d 28. The relative risk reduction was approximately 15% on d 28 and at least 26% on d 14. This translates to a NNT of approximately 6-7 patients on d 14 and 9-10 patients on d 28 (Table 4). The findings of GI endoscopy showed that 20 mg S-pantoprazole was equally effective compared to 40 mg racemic pantoprazole in healing esophagitis (P = 1) and gastric erosions (P = 0.27). Both drugs were well tolerated with no adverse effects. These findings confirm that S-pantoprazole is the more active component of racemic pantoprazole that can be used even at half the dose of racemate to achieve a better efficacy in the treatment of GERD.

Table 5 Findings of GI endoscopy

	Number of patients with findings (n)			
	d 0		d 28	
	Esophagitis	Erosions	Esophagitis	Erosions
Ref	17	6	7	6
Test	21	2	7	3
Fisher's test, P	0.77	0.12	1	0.27

In summary, 20 mg S-pantoprazole is more effective than 40 mg racemic pantoprazole in improving symptoms of heart burn, acid regurgitation, bloating of GERD. Both drugs are safe and well tolerated.

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RAPID COMMUNICATION

Short mucin 6 alleles are associated with *H pylori* infection

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Abstract

AIM: To investigate the relationship between mucin 6 (MUC6) VNTR length and *H pylori* infection.

METHODS: Blood samples were collected from patients visiting the Can Tho General Hospital for upper gastrointestinal endoscopy. DNA was isolated from whole blood, the repeated section was cut out using a restriction enzyme (*Pvu* Ⅱ) and the length of the allele fragments was determined by Southern blotting. *H pylori* infection was diagnosed by ^{14}C urea breath test. For analysis, MUC6 allele fragment length was dichotomized as being either long (> 13.5 kbp) or short (≤ 13.5 kbp) and patients were classified according to genotype [long-long (LL), long-short (LS), short-short (SS)].

RESULTS: 160 patients were studied (mean age 43 years, 36% were males, 58% *H pylori* positive). MUC6 *Pvu* Ⅱ-restricted allele fragment lengths ranged from 7 to 19 kbp. Of the patients with the LL, LS, SS MUC6 genotype, 43% (24/56), 57% (25/58) and 76% (11/46) were infected with *H pylori*, respectively (P = 0.003).

CONCLUSION: Short MUC6 alleles are associated with *H pylori* infection.

Key words: *H pylori*; Mucin 6; Polymorphism; Variable number of tandem repeats

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INTRODUCTION

H pylori has the unique ability to colonize the human stomach. Infection with *H pylori* invariably leads to gastritis and in many instances to peptic ulcer disease[1]. Additionally, *H pylori* infection has been associated with gastric cancer[2]. It is a common infection throughout the world, with prevalence ranging from below 20% in developed countries to over 80% in developing countries. Some risk factors for *H pylori* infection have been identified, such as low socio-economic status or poor hygiene[3]. However, there is remarkable inter-individual variability in susceptibility to the infection that cannot be explained by differences in environmental factors.

Another factor that may be related to *H pylori* infection susceptibility is the composition of the mucus gel layer in the stomach, in which *H pylori* resides. This layer protects the underlying epithelium from acid, proteases, mechanical trauma, and pathogenic micro-organisms and its main constituents are high molecular weight glycoproteins named mucins. These mucins consist of a polypeptide backbone with O-linked oligosaccharide side chains, which largely determine the properties of the mucins[4]. Interestingly, there is substantial inter-individual variation in the number of these side chains. This is caused by a variable number tandem repeat (VNTR) polymorphism in the genes encoding for the mucins. VNTRs consist of repeated DNA sequences and the number of repeats is highly variable. The resulting repeated amino acid sequences are located in the central part of the mucin polypeptide backbone, to which the oligosaccharide side chains are attached[5]. Therefore, this polymorphism leads to the production of mucin polypeptides that substantially differ in both length and glycosylation[6,7]. Thus, this VNTR polymorphism may affect the protective properties of the mucins and consequently the susceptibility to *H pylori* infection.

Normal gastric mucosa is characterized by the expression of mucins MUC1, MUC5AC and MUC6. MUC1 is of the membrane-bound type, whereas MUC5AC and MUC6 are of the secreted, gel-forming type[8]. MUC6 and MUC1 show extensive VNTR variation,

MUC5AC only moderate[6,9]. Furthermore, the length of the repeated sequence differs: a single MUC6 tandem repeat sequence consists of 507 base pairs whereas single MUC1 and MUC5AC tandem repeat sequences consist of only 60 and 24 base pairs, respectively[6]. Therefore, the VNTR polymorphism has the most profound impact on allele length and protein structure of MUC6.

Despite the significant structural consequences of these VNTR polymorphisms, few studies investigated their pathophysiological consequences. In a study comparing gastric cancer patients with healthy blood donors, shorter VNTR sections were associated with gastric cancer for MUC6[10] and MUC1[11]. This effect may be mediated by an altered susceptibility to *H pylori* infection which is an important factor in gastric carcinogenesis, since Vinall *et al*[9] showed that short VNTR sections were associated with *H pylori* infection for MUC1. However, there are no data available regarding the relationship between *H pylori* infection and VNTR polymorphism in MUC6, which is abundant in the stomach and has the most extensive VNTR variation[12].

Therefore, the aim of this study was to investigate the hypothesis that susceptibility to *H pylori* infection is related to MUC6 VNTR length. We studied a sample of 160 patients referred for upper gastrointestinal endoscopy and found that patients with short MUC6 allele fragments have a significantly higher risk of being infected with *H pylori,* which suggests that mucin 6 protein length modifies susceptibility to *H pylori*.

MATERIALS AND METHODS

Subjects

From September to December 2003, all patients with upper gastrointestinal symptoms visiting the Gastroenterology Outpatient Clinic of the Can Tho General Hospital for upper gastrointestinal endoscopy were asked to participate in this study. Patients who had not been treated for *H pylori* infection in the past and who gave written informed consent were included in the study. At baseline, data regarding age, gender, smoking habits and alcohol consumption were registered.

Diagnosis of H pylori infection

All patients had a ^{14}C urea breath test (Heliprobe^{TM}, Noster system AB, Stockholm, Sweden). Patients were not allowed to use proton pump inhibitors/H_2-receptor antagonists or antibiotics during the two weeks preceding the breath testing. After an overnight fast patients took a HeliCap^{TM} capsule (containing 1 μCi of ^{14}C urea) with 50 mL of water. Ten minutes later, a breath sample was collected (BreathCard^{TM}) and analyzed during 4 min. Measuring more than 50 counts was regarded as proof of *H pylori* infection, measuring fewer than 25 counts was regarded as proof of absence of *H pylori* infection[13].

Determination of MUC6 allele length

Blood samples were collected for DNA isolation (Puregene^{TM} kit, Gentra systems, Minneapolis, USA). MUC6 allele fragment length was measured using Southern blot analysis. The DNA samples were digested with the *Pvu*Ⅱ restriction enzyme as described previously by Vinall *et al*[6]. This enzyme cuts just outside the tandem repeat domain and clearly reveals the MUC6 VNTR polymorphism. The resulting DNA fragments were separated by agarose gel electrophoresis (0.7% agarose in 0.04 mol/L Tris, 0.001 mol/L EDTA, pH 8.0) at 35 V for 18 h. We used λ-*Hin*dⅢ digest as a marker of DNA fragment length. DNA fragments were then transferred onto nylon membranes (Gene Screen Plus^{TM} hybridization transfer membrane, Boston, USA). Afterwards, the nylon membranes were treated with ultraviolet radiation and prepared for hybridization by Church buffer. The probe, which consisted of two MUC6 tandem repeats, was produced by polymerase chain reaction with forward primer 5'-ACCTCTTTGGTGACTCCAATTA-3' and reverse primer 5'-AACGTGAGTGGGAAGTGTGGT-3' and randomly labelled with α-^{32}PdCTP. The resulting PCR product was verified by sequencing. After 18 h of hybridization of the probe in 0.5 mol/L phosphate buffer containing 7% SDS and 0.001 mol/L EDTA, SSPE/SDS-solutions were used to remove non-specifically bound probe. Using the λ-*Hin*dⅢ digest as a reference, the individual MUC6 allele fragment lengths were calculated.

Statistical analysis

The primary outcome of this study was the presence or absence of *H pylori* infection. For analysis, MUC6 allele fragment length was dichotomized as being either short (≤ 13.5 kb) or long (> 13.5 kb) and patients were classified according to genotype [long-long (LL), long-short (LS), short-short (SS)]. Baseline characteristics for *H pylori* positive and negative patients were compared. MUC6 genotype and baseline characteristics were related to *H pylori* infection by means of unadjusted and adjusted logistic regression analyses, using the SAS® statistical software package (SAS Institute Inc., USA). Statistical significance was defined as a $P < 0.05$. Missing values were excluded from analysis.

RESULTS

Population

During the study period, 160 patients [mean age 43 years, 58 (36%) males, 92 (58%) *H pylori* infected] were included. Table 1 shows that *H pylori* positive and negative patients were comparable for all baseline characteristics except age.

MUC6 allele fragment length

Using the restriction enzyme *Pvu*Ⅱ, a clear length polymorphism was detected. Minimum fragment length difference was 0.5 kb, reflecting the length of a single MUC6 tandem repeat that consists of 507 base pairs. MUC6 was found to be highly polymorphic with *Pvu*Ⅱ-restricted fragment lengths ranging from 7 to 19 kb [mean 13.8 kb (SD: 2)] (Figure 1).

Factors associated with H pylori infection (Table 2)

Mean MUC6 allele fragment length was shorter for *H pylori* positive patients than for *H pylori* negative patients (13.4

Table 1 Baseline characteristics of *H pylori* positive and negative patients

Characteristic	*H pylori* positive *n* (%) (*n* = 92)	*H pylori* negative *n* (%) (*n* = 68)
Mean age (SD) (yr)	46 (12)[a]	39 (13)
Gender		
Male	29 (50)	29 (50)
Female	63 (62)	39 (38)
Currently smoking		
Yes	22 (56)	17 (44)
No	70 (58)	51 (42)
Current alcohol consumption		
Yes	16 (55)	13 (45)
No	76 (58)	55 (42)

[a]$P < 0.05$ *vs H pylori* negative.

Table 2 Factors associated with *H pylori* infection

Factor	Unadjusted analysis		Adjusted analysis[1]	
	Odds ratio	95% CI	Odds ratio	95% CI
MUC6 genotype (SS *vs* LS/LL)[a]	3.18	1.5-6.9	2.93	1.3-6.5
Age group (> 45 *vs* ≤ 45 yr)[a]	2.11	1.1-4.1	2.11	1.0-4.3
Gender (male *vs* female)	0.62	0.3-1.2	0.47	0.2-1.9
Currently smoking	0.94	0.5-2.0	1.46	0.5-4.3
Current alcohol consumption	0.89	0.4-2.0	1.39	0.5-3.9

[a]$P < 0.05$. [1]Adjusted for MUC6 genotype, age group, gender, current smoking and current alcohol consumption.

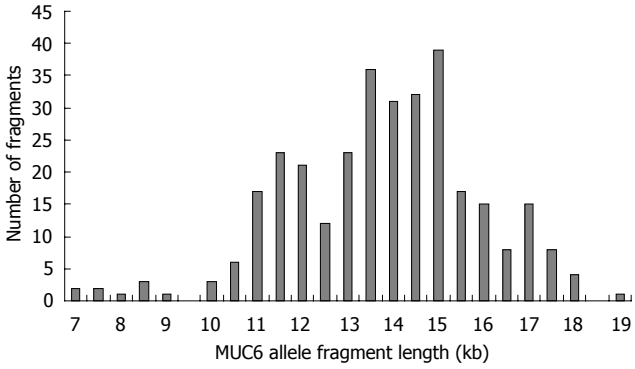

Figure 1 Distribution of MUC6 allele fragment lengths after *Pvu* Ⅱ restriction enzyme digestion.

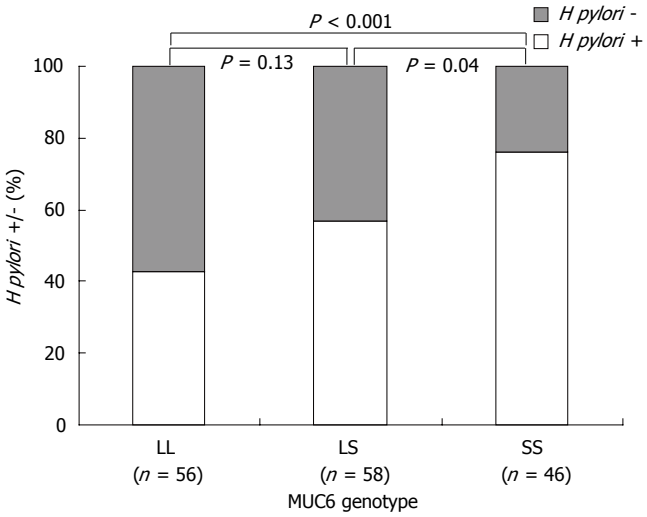

Figure 2 Percentage of *H pylori* infected patients according to MUC6 genotype.

vs 14.2, $P = 0.001$). Since there were too many different allele fragment lengths to analyse separately, MUC6 allele fragment length was dichotomized as being either short (≤ 13.5 kb) or long (> 13.5 kb). Furthermore, patients were grouped according to genotype [long-long (LL), long-short (LS), short-short (SS)].

Patients with two short allele fragments were more often infected with *H pylori* than patients with one long and one short allele fragment [Odds ratio (95% CI): 2.41 (1.0-5.7)] or patients with two long allele fragments [4.24 (1.8-10.0)]. Figure 2 shows that there seems to be a gradual increase in prevalence of *H pylori* infection from 43% (24/56) for patients with two long allele fragments, through 57% (25/58) for patients with one long and one short allele fragments, to 76% (11/46) for patients with two short allele fragments.

Additionally, Table 2 shows that of the other patient characteristics measured, only age was associated with *H pylori* infection, and that the influence of MUC6 genotype remained virtually unchanged after adjusting for age group, gender, smoking and alcohol consumption.

DISCUSSION

The aim of this study was to investigate the relationship between MUC6 VNTR polymorphisms and *H pylori* infection. We were able to confirm that MUC6 VNTR length is highly polymorphic and our data suggest that *H pylori* infection is more frequent in patients with short MUC6 alleles.

Few other studies investigated the MUC6 VNTR polymorphism. Vinall *et al*[6] found 11 different allele fragment lengths for MUC6, ranging from 8 to 13.5 kb. This degree of variation is considerably lower than in our study. It is unlikely that this difference in VNTR length variation is inherent to the Southern blotting technique, as we used the same restriction enzyme. However, the aforementioned study used the Centre d'Étude du Polymorphisme Humain (CEPH) series of families in France and these may be much more homogeneous than our study population.

The relationship between *H pylori* infection and mucin VNTR length has been investigated for another mucin gene, MUC1, and the results were similar to ours. Like other mucins, MUC1 lubricates epithelial structures and constitutes a barrier against acid, proteases and pathogenic organisms. Vinall *et al*[9] showed that short MUC1 alleles were associated with *H pylori*-induced gastritis. Therefore, MUC1 and MUC6, although arising from different families of mucins, may be involved in the same mechanism regarding *H pylori* infection[14].

Our results are also compatible with research focusing

on the relationship between mucin allele length and gastric cancer. In a study investigating 157 gastric cancer patients, it was found that short MUC6 alleles were more frequent in patients with gastric cancer than in healthy blood donors[10]. This seems to be in line with our results that short MUC6 alleles are associated with *H pylori* infection. In fact, because *H pylori* has been classified as a class I carcinogen, the higher prevalence of *H pylori* infection among patients with short MUC6 alleles may (partly) explain the higher prevalence of gastric cancer in these patients. Again, the same goes for MUC1 since Carvalho *et al*[11] stated that short MUC1 alleles were associated with gastric cancer. More research is necessary to determine whether these relationships are independent.

However, other researchers claimed that MUC5AC, and not MUC6, is important in *H pylori* infection: Van den Brink *et al*[15] stated that *H pylori* co-localised with MUC5AC but not with MUC6, and Van de Bovenkamp *et al*[16] stated that MUC5AC, and not MUC6, was the most important receptor for *H pylori*. However, in the study by Van den Brink *et al*, antibodies recognizing the MUC6 precursor rather than mature MUC6 were used. Therefore, it seems plausible that the precursor MUC6 is only found in neck and gland cells, where MUC6 is synthesized. However, the mature MUC6, which is secreted, may be found throughout a much larger area. In fact, Ho *et al*[17] recently confirmed that the mucin within the glands consisted entirely of MUC6, but they also showed that, although the mucus layer on the gastric surface consisted primarily of MUC5AC, layers of MUC6 were interspersed between the layers of MUC5AC.

Regarding the receptor function of the mucins, MUC5AC is the primary source of Lewis B (Le^b), a terminal carbohydrate chain that acts as a ligand for the bacterial adhesion molecule BabA[16,18]. However, although other receptor sites may be present on MUC6[19], mucins may be involved in many other processes besides bacterial binding.

In fact, recently, Kawakubo *et al*[20] showed that secretions from the glands (consisting of MUC6) may have an antibiotic effect on *H pylori,* while secretions from the superficial epithelium (primarily consisting of MUC5AC) may have a pro-biotic effect, thereby limiting *H pylori* infection to the superficial epithelium and protecting the deeper layers of the gastric mucosa. This is consistent with the geographical distribution of *H pylori* described by Van den Brink *et al*[15]. The antibiotic effect was mediated by terminal α-1, 4-linked *N*-acetylglucosamine (α-1,4-GlcNAc) residues, which are present on the variable region of MUC6. Furthermore, the presentation of multiple terminal α-1,4-GlcNAc residues as a cluster may be important for achieving optimal activity. This may explain our finding that shorter MUC6 molecules, which have fewer α-1,4-GlcNAc residues and therefore lower antimicrobial activity, are associated with *H pylori* infection. More research is necessary to further elucidate the functions of the mucins.

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RAPID COMMUNICATION

Resistance to activated protein C is a risk factor for fibrostenosis in Crohn's disease

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Abstract

AIM: To evaluate the effect of resistance to activated protein C (aPCR), the most common known inherited thrombophilic disorder, on the risk of intestinal operation of fibrostenosis in patients with Crohn's disease (CD).

METHODS: In a previous study, we assessed the prevalence of aPCR in CD. In a retrospective case-controlled study, 8 of these CD patients with aPCR were now compared with 24 CD patients without aPCR, matched by gender, age at diagnosis and duration of disease in a 1:3 fashion. The primary end point was the occurrence of an intestinal CD-related operation with evidence of fibrostenosis in the bowel resection specimen.

RESULTS: The Kaplan-Meier analysis revealed that patients with aPCR had a lower probability of remaining free of operation with fibrostenosis than patients without aPCR (P = 0.0372; exact log-rank test) resulting in a significantly shorter median time interval from diagnosis of CD to the first operation with fibrostenosis (32 *vs* 160 mo). At 10 years, the likelihood of remaining free of operation with fibrostenosis was 25% for patients with aPCR and 57.8% for patients without aPCR.

CONCLUSION: CD patients with aPCR are at higher risk to undergo intestinal operation of fibrostenosis than those without aPCR. This supports our hypothesis of aPCR being a possible risk factor for fibrostenosis in CD.

Key words: Fibrostenosis; Resistance to activated protein C; Factor V Leiden; Intestinal surgery; Crohn's disease

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INTRODUCTION

Fibrostenotic lesions of the bowel are frequent and serious complications in Crohn's disease (CD). They often require surgery, which may lead to short bowel syndrome in case of repeated bowel resections[1-4]. Intestinal fibrosis in CD as a consequence of chronic transmural inflammation is characterized by increased production of extracellular matrix, including fibrillar collagen, by activated mesenchymal cells, such as fibroblasts and smooth muscle cells[5,6]. However, the exact pathophysiological mechanism explaining variable disposition of CD patients to develop fibrostenotic lesions is unknown. But some risk factors have been discovered. For example, NOD2/CARD15 variants[7-9] and smoking have been associated with fibrostenosis[10,11]. Moreover, CD patients with antibody responses against *Saccharomyces cerevisiae* (ASCA), CD-related bacterial sequence (I2), *E. coli* outer membrane porin C (anti-OmpC), and neutrophil nuclear antigen (pANCA) have also been reported as being more likely to develop fibrostenotic lesions[12-15].

Resistance to activated protein C (aPCR)[16], representing the most common known inherited thrombophilic disorder[17], has been shown to increase the rate of fibrosis in other inflammatory diseases, such as chronic viral hepatitis[18]. Activated protein C serves as a natural anticoagulant by cleavage and inactivation of factor V a. Factor V plays a central regulatory role in hemostasis, since it contributes to the conversion of prothrombin to active thrombin, which, on the other hand, transforms fibrinogen into fibrin. A single-point mutation in the gene encoding factor V, also known as factor V Leiden, results in a form of factor Va that is resistant to degradation by activated protein C, leading to a relative hypercoagulation state[19].

The increase of the rate of fibrosis in chronic viral hepatitis caused by aPCR was explained by thrombotic obliterations of small portal and hepatic veins resulting in local hepatocyte death and increased development of fibrosis and by the effect of thrombin through activation of stellate cells, which are the mediators of fibrosis in the liver[20]. However, the role of aPCR in the development of fibrostenotic lesions in CD has not been elucidated till now. The potential role of the clotting system in CD has already been shown. Granulomatous vasculitis, intravascular fibrin deposition and capillary microthrombi have been reported in CD lesions[21,22]. Additionally, CD has been observed less frequently in patients with inherited haemophilic disorders[23]. On the other hand, aPCR as a thrombophilic disorder may favor intravascular fibrin deposition and thrombotic obliteration of the small vessels of intestinal lesions in CD, resulting in increased local cell death and fibrosis.

We, therefore, hypothesized that aPCR increases the rate of fibrosis in CD patients. We expected that CD patients with aPCR are more likely to have fibrostenotic lesions of the bowel than patients without aPCR, resulting in a lower probability of remaining free of operation with fibrostenosis.

MATERIALS AND METHODS

Patients and study design

This is a retrospective case-control study on the influence of aPCR on the presence of fibrostenotic lesions found at intestinal CD-related operations in CD patients. In a previous study on risk factors for thromboembolism in inflammatory bowel disease (IBD), we assessed the prevalence of aPCR in patients with IBD, including 77 patients with CD[24]. We had detected 8 CD patients with aPCR who were now compared with CD patients without aPCR selected from the same database. CD patients with aPCR (n = 8) and without aPCR (n = 24) were matched by gender, age at diagnosis (± 10 years) and duration of disease (± 3 years) in a 1:3 fashion. A colleague blinded for the clinical course of the patients performed the matching. All patients included in the study were Caucasians and had been in routine care at our institution (Medical University of Vienna, Department of Internal Medicine IV, Division of Gastroenterology and Hepatology, Vienna). The diagnosis of CD was based on established criteria of clinical, radiological, endoscopic, or histological findings[25]. The location of CD was determined according to the Vienna classification[26]. The information on the clinical course, smoking habits, and the medical and surgical management was reviewed from the charts of the patients. A "smoker" was defined as a patient who had smoked at least seven cigarettes weekly for at least one year[11]. Immunosuppressants, such as azathioprine, were considered for analysis if the duration of treatment had been at least 3 mo.

The primary end point of the study was the presence of fibrostenosis found at intestinal CD-related operations documented by the pathological and/or surgical report, respectively. The secondary end point was the occurrence of intestinal CD-related operations regardless of fibrostenosis. Only intestinal operations, such as bowel resections, strictureplasties and gastrointestinal bypass-surgery, were included in the analysis. Creations of a stoma, intestinal reconstructions, exploratory laparatomies, and surgery of complicated perianal CD were excluded from the analysis. Two observers blinded for the aPCR status of the patients (W.R. and J.P.) reviewed the pathological and surgical reports of the patients for the presence of fibrostenosis. The Ethics Committee of the Medical University of Vienna approved this study.

Definition of fibrostenotic lesions

Stricturing disease is defined by the Vienna classification as the occurrence of constant luminal narrowing demonstrated by radiologic, endoscopic, or surgical examination combined with prestenotic dilatation and/or obstructive signs or symptoms but without evidence of penetrating disease[26]. In our retrospective analysis, for the purpose of defining fibrostenotic lesions at the time of surgery, we modified this definition for four reasons. First, strictures of the bowel in CD may result not only from fibrostenotic lesions but also from inflammatory obstruction without fibrosis. Second, internal fistulas are often associated with strictures and such mechanical factors may even favor the development of fistula, which might explain the coexistence of fibrostenotic lesions and perforation[27]. Third, radiologic, endoscopic, and surgical examination may report contradictory results. The most reliable data on complicated disease are available after resection from surgical and pathological reports. In these reports, fibrostenotic lesions were defined as luminal narrowing and bowel wall thickening on naked-eye examination of the surgical resection specimen. Histologically, strictures were recognized by thickening of the muscularis mucosa and by fibrosis of the submucosa[27]. And fourth, we excluded "obstructive symptoms" from the definition, since it may be difficult to differentiate clinically from other disease-related complications (e.g. inflammation, fistula and abscess).

We, therefore, defined fibrostenotic lesions as the occurrence of bowel wall thickening and luminal narrowing on naked eye examination in the pathological and surgical report found at intestinal CD-related surgery as well as the occurrence of thickening of the muscularis mucosa and of fibrosis of the submucosa in the histological part of the pathological report. In case of discrepancies between pathological and surgical examination, the pathological report of the surgical resection specimen was rated with higher priority than the surgical report. In case of strictureplasties and gastrointestinal bypass surgery, if a surgical resection specimen was not available, the assessment of fibrostenosis was based solely on the surgical report. The diagnosis of a fibrostenotic lesion was irrespective of evidence of penetration.

Assay system

aPCR was determined as described previously using the assay Coatest® aPC™ Resistance (Chromogenix, Mölndal, Sweden) according to the manufacturer's instructions[24]. The normal range of the aPC ratio was > 1.9 and aPCR, therefore, was diagnosed if the aPC ratio was ⩽ 1.9. This

Table 1 Characteristics of 32 patients with Crohn's disease (frequencies, %, median values with range)

	With aPCR (n = 8)	Without aPCR (n = 24)
Sex ratio (M/F)	3/5	9/15
Age at diagnosis (yr)	30 (13-53)	31 (10-46)
Duration of disease (mo)	140 (86-257)	145 (65-272)
aPC ratio[1]	1.52 (1.19-1.64)	2.31 (2.08-2.66)
Patients with VTE in the history	5 (63%)[b]	2 (8%)
Smokers	4 (50%)	19 (79%)
Patients under azathioprine	4 (50%)	15 (63%)
Location of CD[2]		
Terminal ileum	2 (25%)	4 (17%)
Colon	1 (12.5%)	5 (21%)
Ileocolon	4 (50%)	12 (50%)
Upper gastrointestinal tract	1 (12.5%)	3 (12%)

aPCR: resistance to activated protein C; CD: Crohn's disease; VTE: venous thromboembolism. [1]The patients were divided into the two groups according to the value of the aPC ratio: ≤ (diagnosis of aPCR) and > 1.9 (normal range), respectively. [2]The location of disease was classified according to the Vienna classification[26]. [b]$P < 0.01$ *vs* patients without aPCR.

Table 2 Intestinal CD-related surgery in 32 patients with Crohn's disease (frequencies, %, median values)

	With aPCR (n = 8)	(n = 24)
Patients having undergone an operation[1]	6 (75%)	16 (67%)
Patients having undergone an operation with fibrostenosis[2]	6 (75%)	13 (54%)
Patients with fibrostenosis at 1st operation[2]	6/6 (100%)	10/16 (62.5%)
Location of fibrostenosis at 1st operation[3]		
Terminal ileum	4	8
Colon	2	0
Upper gastrointestinal tract	0	2
Median time from diagnosis of CD to 1st operation[1] (mo)	32	80.5
Median time from diagnosis of CD to 1st operation with fibrostenosis[2] (mo)	32[a]	160

aPCR: resistance to activated protein C; CD: Crohn's disease. [1]Operations: intestinal CD-related operations; [2]Operations with fibrostenosis: intestinal CD-related operations with fibrostenosis; [3]The location of fibrostenosis was classified according to the Vienna classification for location of Crohn's disease[26]. [a]$P < 0.05$ *vs* patients without aPCR.

test with predilution of samples with factor V-deficient plasma has been shown to have a very high sensitivity and specificity of nearly 100% for the diagnosis of factor V Leiden, including a discrimination between heterozygous and homozygous subjects[28-30].

Statistical analysis

All computations were performed with the use of SAS software, version 9 (SAS Institute Inc., Cary, NC, USA). Data were presented as frequencies, percentages and median values with range, respectively. Differences between groups were analyzed using the fisher's exact test for categorical data and the Mann-Whitney *U*-test for continuous data. Survival time methods were used to analyze the time from diagnosis of CD to the first intestinal CD-related operation with fibrostenosis and from diagnosis of CD to the first intestinal CD-related operation, respectively, (uncensored observations) or the duration of follow-up among patients without intestinal CD-related operation with fibrostenotic lesions and without intestinal CD-related operation, respectively (censored observations)[31]. The probability of survival free of intestinal CD-related operation with fibrostenosis and survival free of intestinal CD-related operation regardless of the presence of fibrostenosis, respectively, was estimated according to the Kaplan-Meier method[32]. Differences between Kaplan-Meier curves of both groups were analyzed using the exact log-rank test for unequal follow-up[33]. The study design included 1:3 matching (CD with aPCR : CD without aPCR) with regard to gender, age at diagnosis and duration of CD. Differences were considered significant if P was < 0.05.

RESULTS

Study population

The clinical data of both patient groups are summarized in Table 1. Eight patients had an aPC ratio below 1.9 and were diagnosed as having aPCR: one patient had an aPC ratio of 1.19 consistent with homozygous for factor V Leiden and the other 7 patients had aPC ratios ranging from 1.46 to 1.64 consistent with heterozygous for factor V Leiden[30]. Twenty-four patients had values of the aPC ratio within the normal range. Neither patient group differed in terms of location of disease, and percentage of smokers and patients who had been treated with azathioprine (Table 1). Furthermore, the number of cigarettes per day, the duration of smoking, and the dosage and duration of treatment with azathioprine did not differ between either patient group (data not shown). No immunosuppressants other than azathioprine had been administered. None of the patients had received infliximab or any other biological agent. Patients with aPCR had a history of venous thromboembolic complications diagnosed by imaging procedures significantly more often than the patients without aPCR ($P < 0.01$). Most of the thromboembolic events had been deep venous thromboses of the legs and pulmonary emboli.

Intestinal CD-related surgery

Six of 8 patients with aPCR and 16 of 24 patients without aPCR underwent intestinal surgery. Altogether, 44 intestinal CD-related operations (13 in patients with aPCR and 31 in patients without aPCR) had been performed. All operations had been intestinal resections, except for one case of gastroentero-anastomosis, which was necessary to circumvent the obstructed duodenum in a patient with CD of the upper gastrointestinal tract. A strictureplasty and a resection of another bowel segment performed at the same surgical procedure in a patient with aPCR were counted as one operation. The location of the bowel resections did not differ between both patient groups and was most likely of the terminal ileum (Table 2). In addition to bowel resections, three balloon dilations of fibrostenotic

lesions had been performed in two patients with aPCR but not in patients without aPCR. These interventions were not included in the analysis.

Operations with fibrostenosis: primary end point

There was a clear tendency for fibrostenosis to be found more often in the presence of aPCR. At the first intestinal CD-related operation, fibrostenotic lesions were found in all 6 (100%) patients with aPCR, but only in 10 out of 16 (62.5%) patients without aPCR ($P = 0.079$). The Kaplan-Meier analysis revealed that patients with aPCR had a significantly lower probability of remaining free of operation with fibrostenosis compared to patients without aPCR ($P = 0.0372$; exact log-rank test). This was also represented by the median time from diagnosis to the first operation with fibrostenosis being significantly shorter in patients with aPCR than in patients without aPCR (32 *vs* 160 mo; Table 2). In a further step, the calculation was limited to 120 mo, since this time interval was just below the median observation time of the patients (140 and 145 mo, respectively) and, therefore, more than half of the patients were under follow-up during the first 10 years after diagnosis of CD ($P = 0.0216$; exact log-rank test) (Figure 1). At 10 years, the likelihood of remaining free of operation with fibrostenosis was 25% for patients with aPCR and 57.8% for patients without aPCR.

Both observers who reviewed the pathological and surgical reports of the patients for fibrostenotic lesions agreed on the presence or absence of fibrostenosis in all operations. In 4 out of 44 operations, a fibrostenosis was described in the pathological report but was not mentioned in the surgical report. In all these four cases (1 patient with aPCR and 3 patients without aPCR), the surgeons described inflammatory masses that made it difficult to diagnose fibrostenosis in the surgical situation.

Overall operations: secondary end point

Concerning any intestinal CD-related operation without regard to whether it revealed fibrostenosis or not, patients with aPCR did not undergo operations more often than patients without aPCR. Six of 8 (75%) patients with aPCR and 16 of 24 (67%) patients without aPCR had undergone intestinal surgery ($P = 0.32$) (Table 2). Furthermore, the median time interval from diagnosis of CD to the first operation (Table 2), and the probability of remaining free of intestinal surgery (Kaplan-Meier survival curve not shown) did not significantly differ between the two patient groups ($P = 0.24$).

DISCUSSION

To our best of knowledge, this is the first report on an association between resistance to activated protein C and fibrostenosis in Crohn's disease. In a case-controlled study setting, aPCR increased the risk of fibrostenotic lesions found at intestinal surgery as described by surgical and/or pathological reports. CD patients with aPCR had a significantly lower probability of remaining free of intestinal operations with fibrostenosis. These data support our hypothesis that aPCR accelerates the development of fibrosis in intestinal lesions in CD patients.

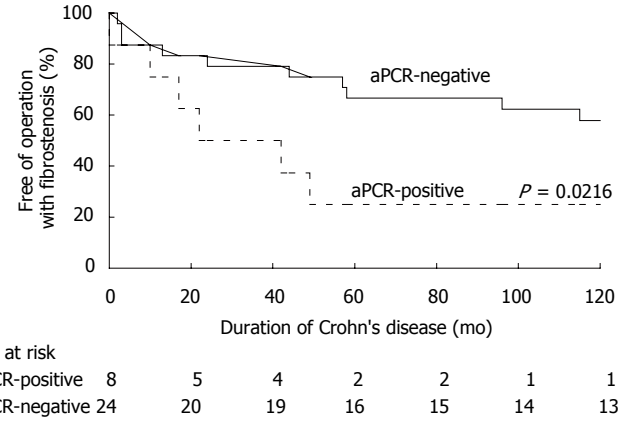

Figure 1 Kaplan-Meier curves for CD patients with and without aPCR, for remaining free of intestinal CD-related operation with fibrostenosis. A significantly lower proportion of patients with aPCR remained free of operation with fibrostenosis as compared to CD patients without aPCR ($P = 0.0216$; exact log-rank test). The calculation was limited to a period of 120 mo.

The primary end-point in the present study was the occurrence of an intestinal CD-related operation with fibrostenosis. Fibrostenotic lesions were referred to bowel wall thickening and luminal narrowing on naked-eye examination and to patho-histological features. This definition of fibrostenosis was modified from the definition of disease behavior used in the Vienna classification[26] and was irrespective of evidence of penetration. The most important reasons for modification were the association of internal fistula and fibrostenosis in multiple bowel resection specimens[27] as well as the possible occurrence of inflammatory bowel obstruction without fibrostenosis. It is difficult or sometimes impossible to distinguish inflammatory from fibrostenotic strictures by current diagnostic techniques, including imaging procedures and endoscopy, leaving the question of what is the gold standard for the differential diagnosis? Is there any method, which is superior to direct visualization by the surgeon and the pathologist as well as to patho-histological investigation? From the literature, good evidence cannot be retrieved. Thus, we based our evaluation of fibrostenosis on the assumption that surgical and pathological reports deliver the most reliable data on the presence of fibrostenosis and are, therefore, superior to imaging procedures in the diagnosis of fibrostenotic strictures on expert opinion.

aPCR is the most common known inherited thrombophilic disorder known until now[16,17]. The genetic basis of aPCR is a single-point mutation in the gene encoding for coagulation factor V, also known as factor V Leiden. Factor V Leiden has a 2%-7% prevalence in most Caucasian populations and is identified in 20%-50% of patients with venous thromboembolism[17,34-36].

In addition to the increased risk of venous thromboembolism, aPCR and other thrombotic risk factors have also been shown to accelerate the rate of fibrosis in inflammatory diseases, such as chronic viral hepatitis[18,37,38]. Thrombotic obliterations of small portal and hepatic veins and activation of the stellate cells by

thrombin were assumed to cause increased fibrosis in the liver[20]. The effect of thrombin is mediated through activation of thrombin receptors on the stellate cells, which are up-regulated during chronic liver injury[39]. Furthermore, aPCR significantly increased pulmonary fibrosis in bleomycin-induced inflammatory lung injury in mice[40]. Additionally, an association between aPCR and fibrosis has been described in a transgenic factor V Leiden mouse model with enhanced fibrin deposition in multiple tissues[41]. In CD, an influence of the clotting system on the pathomechanism has been assumed based on the finding of intravascular fibrin deposition and capillary microthrombi[21,22] and a reduced frequency of CD in patients with inherited haemophilic disorders[23].

The association between aPCR or any other thrombotic risk factor and fibrosis in CD has not been previously investigated. An increased rate of fibrosis may lead to fibrostenotic lesions of the bowel, which often require surgery. Intestinal fibrosis and fibrostenosis are caused by uncontrolled proliferation of mesenchymal cells and excessive production of extracellular matrix proteins, including fibrillar collagen[5,6]. Fibroblasts cultured from fibrostenotic lesions in CD patients, for instance, have been shown to synthesize an increased amount of collagen type Ⅲ[42]. A precondition for the development of fibrosis is a chronic transmural inflammation, which is a typical feature of CD. Infiltrating immune cells are considered to secrete cytokines and growth factors, such as transforming growth factor beta (TGF-β), in the mesenchymal layers, which might lead to activation of fibroblasts[6,43].

Some risk factors predispose to fibrostenosis in CD. Several studies reported that CD-associated NOD2/CARD15 variants are associated with fibrostenotic lesions[7-9] and, furthermore, that CD patients who are positive for ASCA, I2, anti-OmpC or pANCA are also more likely to develop fibrostenosis[12-15]. Additionally, smoking has been shown to predispose to ileal localization of CD and to fibrostenotic lesions[10-11]. But for all the itemized risk factors, it remains to be determined whether the association with fibrostenosis is independent or secondary to association with ileal involvement.

Immunosuppressive drugs do not seem to prevent the formation of fibrostenosis. Azathioprine has been shown to improve mucosal healing but not the development of fibrostenosis in CD, especially in the ileocecal region[44-46]. A recent retrospective study on the use of immunosuppressive drugs and the need for intestinal surgery described only a tendency of reduced probability of formation of fibrostenosis over the time, despite a significant increase of the use of azathioprine in the same time period[4]. However, the only immunosuppressive drug given to the patients in the present study was azathioprine, which was equally frequently administered to the patients of both groups. We can, therefore, exclude any influence of medical treatment on the results of our study.

Furthermore, there was no difference in smoking habits between the two patient groups. But all the other risk factors for fibrostenosis, such as NOD2/CARD15 variants, ASCA, I2, anti-OmpC, and pANCA, were not fully available and, therefore, not included in the analysis. It was thus not possible to investigate the influence of these risk factors on our results. A further limitation might be the small number of patients included in the study. Since the effect of aPCR on fibrostenosis in CD was unknown prior to the present investigation, a calculation of the sample size was not possible. Our aim was to perform a pilot study on an association between aPCR and fibrostenosis in CD and by this way to generate the basis for a working hypothesis.

aPCR did not significantly influence the number of overall intestinal CD-related operations regardless of fibrostenosis. Therefore, we have no evidence that other indications for surgical treatment in CD except fibrostenotic strictures are influenced by aPCR.

In summary, our results give rise to this hypothesis that aPCR may be a risk factor for fibrostenosis in CD. The effect of aPCR might not be specific and coagulation might generally be involved in the development of fibrosis in CD, disposing patients with any thrombophilic disorder also to have an increased tendency to fibrostenosis. We are aware that our data should be verified by further larger trials, including data about other known risk factors for fibrostenosis. However, our study contains the first reference to an association between aPCR and fibrostenosis in CD and may provide a new insight in the pathogenesis of fibrostenosis in CD.

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RAPID COMMUNICATION

Xeroderma pigmentosum group D 751 polymorphism as a predictive factor in resected gastric cancer treated with chemo-radiotherapy

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Abstract

AIM: To evaluate the potential association of xeroderma pigmentosum group D (XPD) codon 751 variant with outcome after chemo-radiotherapy in patients with resected gastric cancer.

METHODS: We used PCR-RFLP to evaluate the genetic XPD *Lys751Gln* polymorphisms in 44 patients with stage Ⅲ (48%) and Ⅳ (20%) gastric cancer treated with surgery following radiation therapy plus 5-fluorouracil/leucovorin based chemotherapy.

RESULTS: Statistical analysis showed that 75% (12 of 16) of relapse patients showed *Lys/Lys* genotype more frequently (P = 0.042). The *Lys* polymorphism was an independent predictor of high-risk relapse-free survival from Cox analysis (HR: 3.07, 95% CI: 1.07-8.78, P = 0.036) and Kaplan-Meir test (P = 0.027, log-rank test).

CONCLUSION: XPD *Lys751Gln* polymorphism may be an important marker in the prediction of clinical outcome to chemo-radiotherapy in resected gastric cancer patients.

Key words: Xeroderma pigmentosum group D gene; Polymorphism; Gastric cancer; Radiotherapy

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INTRODUCTION

The xeroderma pigmentosum group D (XPD) gene encodes a protein required for nucleotide excision repair (NER). This product recognizes and repairs a wide range of structurally unrelated lesions such as bulky adducts caused by UV light, environmental agents, cross links and oxidative damage[1,2]. Moreover, XPD gene is one of the components of basal transcription factor IIH (TFIIH), participating also in transcription initiation. Since XPD is involved in both transcription and NER, it may be able to repair other types of damage, such as radiation therapy-induced damage. Because XPD interacts with many different proteins as part of TFIIH transcription factor, amino acid variants in different domains of XPD, such as 683 and 751, may affect different protein interactions, and result in expression of different phenotypes[3,4].

Intrinsic and acquired resistance to cancer chemotherapeutic agents results from polymorphisms in genes encoding DNA repair enzymes. Differences in responsiveness of cancer cells to anticancer agents can be differently affected by changes in repair efficiency. Clinical outcomes after chemotherapy may be influenced by pharmacogenetic polymorphisms in DNA repair enzymes. In this sense, three single nucleotide polymorphisms (SNP) in the XPD gene (in codons 156, 312, and 751) are related with different DNA repair capacity[5-7]. Thus, variants generated by amino acids can change *Asp312Asn* (exon 10, G > A substitution) and *Lys751Gln* (exon 23 A > C substitution) located in conserved regions of the XPD protein, and are associated with lower efficiency of damage repair and significantly higher background frequency of apoptotic cells in irradiated lymphocytes[8].

Although gastrectomy is the only potentially curative treatment in gastric-cancer patients, the overall survival results remain unsatisfactory. The main factor accounting for high mortality is relapse after surgical resection. During the past few decades, the principle of combined treatment modality has been developed and applied in various solid tumors including gastric cancer. In order to prevent recurrence and increase the cure rate of gastric-cancer after surgery, multiple studies using variable treatment modali-

ties have been undertaken. One of the landmark studies reported that chemo-radiotherapy can significantly improve survival after resection of stage IB to stage Ⅳ gastric cancers[9]. Chemo-radiotherapy has been increasingly recognized as a standard of care since then[10,11]. However, whether adjuvant chemo-radiotherapy can prolong the survival of patients with extensive lymph node dissection remains debatable. In this paper, we evaluated the potential role of XPD codon 751 variant in the outcome of 44 patients with resected gastric cancer after chemo-radiotherapy.

MATERIALS AND METHODS

Patients

Characteristics of the patients are listed in Table 1. From October 1992 to January 1999, 44 patients consisting of 32 men (73%) and 12 women (27%) with a median age of 60 years (range: 33-77 years) with diagnosis of gastric cancer after having undergone gastrectomy were treated with radiation plus 5-fluorouracil (5-FU) and leucovorin. Thirty-two percent of the patients had stage Ⅰ - Ⅱ and 68% stage Ⅲ-Ⅳ gastric cancer at the time of diagnosis (Table 1). The median follow-up time was 46.9 mo (range: 5.33 to 124.06 mo). The median time to progression was 37.5 mo (range: 2.3 to 95.4 mo). This study was conducted in Navarra Hospital Center, and informed consent was obtained from all the patients for using their tissues. The clinicopathological information of each subject was obtained from the tumor registry at Navarra Hospital.

Table 1 Characteristics of the patients

Baseline factors	*n* (%)
Sex	
Male	32 (73)
Female	12 (27)
Age (yr)	
⩽ 50	16 (36)
51-64	13 (30)
⩾ 65	15 (34)
Median (Range)	60 (33-77)
Stage at diagnostic	
Ⅰ - Ⅱ	14 (32)
ⅢA + ⅢB	21 (48)
Ⅳ	9 (20)
Histotype	
Intestinal	9 (20)
Diffuse	35 (80)
Grading	
Ⅰ	3 (7)
Ⅱ	8 (18)
Ⅲ	33 (75)
Tumor location	
Cardia	9 (21)
Fundus	9 (21)
Body	10 (24)
Antrum	14 (33)
Gastrectomy	
R0	35 (80)
R1	8 (18)
R2	1 (2)
XPD 751	
Lys/Lys	19 (51)
Lys/Gln	14 (38)
Gln/Gln	4 (11)
Unknown	7

Treatment schedule

The patients received postoperative treatment with 5-FU plus leucovorin and local radiation 20-40 d after gastrectomy. This chemotherapy regimen developed by the North Central Cancer Treatment Group[12] was used before and after radiation. Chemotherapy (fluorouracil, 425 mg/m^2 per day, and leucovorin, 20 mg/m^2 per day) was initiated on d 1 followed by chemo-radiotherapy on d 28 after the initial cycle of chemotherapy. Chemo-radiotherapy consisted of 4500 cGy of radiation at 180 cGy/d, 5 d/wk for 5 wk, with 5-FU (400 mg/m^2 per day) and leucovorin (20 mg/m^2 per day) on the first four and the last three days of radiotherapy. One month after radiotherapy, two 5-d cycles of 5-FU (425 mg/m^2 per day) plus leucovorin (20 mg/m^2 per day) were given every other month.

The 4500 cGy of radiation was delivered in 25 fractions (5 d/wk) to the tumor bed, regional nodes and 2 cm beyond the proximal and distal margins of resection. The adjuvant treatment was performed as previously described[9].

Samples and DNA extraction

Surgical specimens (paraffin blocks) were cut into 5 μm-thick sections for molecular analysis.

Three tissue sections were transferred into a micro centrifuge tube and 1.2 mL of xylene was added. After centrifugation at 14 000 r/min for 5 min at room temperature (RT), the supernatant was removed. Subsequently, the tissue samples were washed in 1.2 mL of 96 mL/L ethanol. After centrifugation at 14 000 r/min for 5 min at RT, the supernatant was discarded. The washing procedure was repeated another time. The samples were dried for 3-5 min, in a vacuum pump (any letter between were and dried).

DNA was extracted using the QIAamp DNA Mini Kit (Qiagen-IZASA- Barcelona Spain). The deparaffinized samples were re-suspended in 180 μL of ATL buffer plus 20 μL of proteinase K and incubated overnight at 56℃. The samples were processed according to the provided protocol. The purified DNA was finally eluted in a total volume of 150 μL. DNA yield was quantified by NanoDrop 3.0.0 (Nucliber, Wilmington, Delaware USA).

About 7-509 mg/L of DNA was extracted and 45-200 ng of genomic DNA was used as template. PCR/RFLP-based assays were performed as described previously[5] with slight modifications. The PCR product (20 μL) was digested with 25 units of Pst I enzyme (New England BioLabs, -IZASA, S.A, Barcelona-, Spain) in 50 μL reaction mixture for 1 h. The digestion product was visualized in 3% agarose gel (Pronadisa, Madrid, Spain). The wild-type homozygote was defined by 104- and 220-bp banding patterns; the heterozygote by 63-, 104-, 157-, and 220-bp fragments; and variant homozygote

Table 2 Toxicity evaluated

Toxicity grade Ⅲ-Ⅳ	*n* (%)
No	28 (64)
Yes	16 (36)
Toxicity type	
Hematological	10 (62)
Digestive	3 (19)
Others	3 (19)

Table 3 Relapse site

Relapse	*n* (%)
No	24 (54.5)
Yes	20 (45.5)
Relapse sites	
Local	5 (25)
Distant	10 (50)
Local + Distant	5 (25)

by 63-, 104-, and 157-bp fragments. Some samples were noted as "unknown" because they could not be amplified due to a relatively frequent occurrence of PCR inhibitory substances in samples prepared with this DNA extraction method (Table 1). Furthermore, three DNA samples corresponding to each genotype selected by direct sequencing were used. The results were in concordance with RFLP genotyping.

Statistical analysis

This study was designed to analyze the role of XPD polymorphism in the prediction of relapse. Relapse free-survival (RFS) was defined as the time from the start of chemotherapy to the first evidence of disease progression. RFS was calculated using the Kaplan-Meier method. Contingency tables and chi-square test (χ^2) were used to summarize the association of relapse with XPD polymorphism. All *P* values were two-sided. Cox proportional hazards models were also used to evaluate the different variables considered. All statistical tests were conducted by SPSS software 11.0 version for Windows (SPSS, Inc. Chicago). $P \leqslant 0.05$ was considered statistically significant.

RESULTS

Thirty-seven patients were evaluated for the *Lys751Gln* polymorphism. Seven patient were not assessed due to poor sample extraction quality. Fifty-one percent (19 of 37) of the patients were homozygous for the *Lys/Lys* genotype, 38% (14/37) heterozygous for *Lys/Gln,* and 11% (4 of 37) homozygous for the glutamine variant (Table 1). The *751Gln* allele frequency was 0.33, similar to that observed in other studies[5,7].

Sixteen patients (36%) developed grade Ⅲ-Ⅳ toxicity. Hematological toxicity was found in 62% (10/16) patients compared with gastrointestinal and other toxicity in19% (3/16) patients (Table 2). However, there was no association between XPD genotype and toxicity (data not shown).

Table 4 Results of chi-square test for disease relapse

Relapse	XPD			Stage	
	Lys	*Lys/Gln*	*Gln/Gln*	Ⅰ + Ⅱ	ⅢA + ⅢB + Ⅳ
No	7	11	3	11	13
Yes	12	3	1	3	17
P		0.042		0.050	

Table 5 Unadjusted XPD genotype and relapse-free survival (RFS)

XPD	RFS (median)	Hazard Ratio	95% CI
Lys/Lys	11.79	1.0	Reference
Lys/Gln	14.33	0.35	0.11 to 1.09
Gln/Gln	23.33	0.25	0.03 to 1.96

Table 6 Adjusted Cox multivariate analysis for RFS

XPD	RFS (median)	Hazard ratio	95% CI	*P*
Lys/Lys	11.79	3.07	1.07 to 8.78	0.036
Lys/Gln + Gln/Gln	20.26	1.0	Reference	

Gastrectomy with D1 lymphadenectomy was performed in all patients. Of the 44 patients, 20 (45.5%) had relapse and 17 (85%) of them died. The first relapse site was local in 5, distant in 10, and both local and distant in 5 (Table 3).

Statistical analysis showed that 75% (12 of 16) of relapse patients showed *Lys/Lys* genotype more frequently ($P = 0.042$, Table 4).

Other clinico-pathological features such as histology, grading and localization were not significant (data not shown).

The relative risk of progression (with *Lys/Lys* genotype used as the reference) was 0.35 (95% CI: 0.11-1.09) for patients carrying the *Lys/Gln* genotype and 0.25 (95% CI: 0.03-1.96) for the *Gln/Gln* group (Table 5).

Only XPD genotype and stage to diagnosis showed a significant relation with cancer relapse and were evaluated as potential predictors of RFS by Cox test. We used the combined genotypes *LysGln/GlnGln* as reference in these analyses and found that the *Lys/Lys* genotype was more strongly associated with disease progression ($P = 0.036$) than the combined *LysGln/GlnGln* genotype (adjusted HR: 3.07, 95% CI: 1.07-8.78) (Table 6, Figure 1).

DISCUSSION

Most cases of gastric cancer are diagnosed at an advanced stage with poor prognosis. Surgery remains the only potentially curative treatment, but it is associated with a high rate of local recurrence and distant metastases. When irradiation is combined with surgical resection for all or a

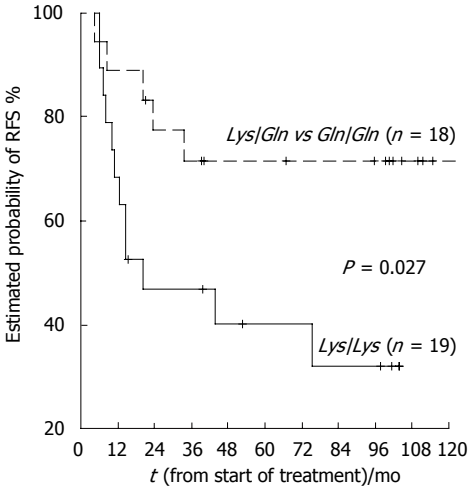

Figure 1 *XPD*751 polymorphism and RFS under radio-5Fu/Lv treatment.

majority of tumors, both survival and local control appear to be better than those for the unresected patients[13]. Epidemiological studies have shown that *Gln* variant at *XPD-751* polymorphism is partiality able to repair DNA in lung cancer[7,14]. In this sense, cancer treatment is to cause DNA damage and tumor cell apoptosis. *Gln* variant seems to show a lower ability to repair DNA damage and cancer patients are more sensitive to chemo-radiotherapy. In contrast, the presence of allele *Lys* represents a positive effect in different DNA repair pathways and a possible poor response to cancer treatment. In this study, we found a significant relationship between clinical response to chemo-radiotherapy and the XPD *Lys751Gln* polymorphism. Patients with the *Lys/Lys* genotype were more likely to have relapse compared to those with the combined *Lys/Gln* and *Gln/Gln* genotype.

Scientific publications are available but with diverging results in the *XPD751* polymorphism and cancer risk or treatment efficiency[3,5-7]. Thus, decreased DNA repair has been associated with *Lys* allele[6]. Epidemiological study has reported association of this allele with a higher risk of basal cell carcinoma[5] even though no significant relationship between this polymorphic gene and DNA repair proficiency has been reported[15,16]. Spitz *et al*[7] and other research group[17] reported a suboptimal DNA repair capacity (DRC) particularly in subjects who were homozygous for *751Gln* alleles. Moreover, a recently study has found a tendency toward a higher background frequency of apoptotic cells in irradiated lymphocytes carrying variant homozygote *Gln* at XPD codon 751[8]. Our results are in agreement with these studies, suggesting that heterozygote and homozygote *Gln* alleles may therefore increase their clinical response to suboptimal DNA damage repair after treatment.

Repair of DNA damage is a complex process[1,18,19]. Functional protein of XPD could be affected by a structural change (*Lys* → *Gln*) from a basic to a polar amino acid change located at about 50-base upstream from the poly (A) signal[5]. Moreover, close proximity in the genome between XPD and other polymorphic DNA repair genes such as X-ray repair cross complementing (XRCC1) and excision repair cross complementing (ERCC1) may influence the interaction between them leading to different or synergic DNA repair capability. A recent molecular epidemiology study has genotyped 44 SNPs in 20 genes involved in four DNA damage repair pathways, showing that homozygous *Gln751Gln* and other gene variants genotyped are highly associated with lung cancer risk[20]. In the same way, variant *Gln* allele and some nucleotide and base excision repair (NER and BER, respectively) variant gene members are associated with increased level of polycyclic aromatic hydrocarbon-DNA adducts[15,21].

In conclusion, XPD *Lys751Gln* polymorphism may be an important marker of genotoxicity that can predict the clinical outcome of resected gastric cancer treated with chemo-radiotherapy.

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RAPID COMMUNICATION

Perinatal events and the risk of developing primary sclerosing cholangitis

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Abstract

AIM: To investigate whether perinatal events, intrauterine or postpartum, are associated with the development of primary sclerosing cholangitis (PSC) later in life.

METHODS: Birth records from 97 patients with adult PSC in Sweden were reviewed. Information on perinatal events including medications and complications during pregnancy, gestation length, birth weight and length were collected. Two control children of the same sex were selected for each subject. Conditional multiple logistic regression was used to assess associations of the perinatal measures with development of PSC.

RESULTS: No significant associations were found between gestational age, birth length, breastfeeding, and the majority of medical complications including infections or medication during pregnancy for the mothers or postpartum for the children. Vaginal bleeding and peripheral oedema showed associations with PSC, with matched odds ratios of 5.70 (95% CI, 1.13-28.83) and 2.28 (95% CI, 1.04-5.03), respectively.

CONCLUSION: The associations of vaginal bleeding and oedema with subsequent PSC cannot readily be explained, so our findings do not strongly support the hypothesis of a significant role of perinatal events as a risk for the development of PSC later in life.

Key words: Inflammatory bowel disease; Perinatal factors; Sclerosing cholangitis

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INTRODUCTION

Primary sclerosing cholangitis (PSC) is a complex disease likely to involve multiple susceptibility genes, environmental and immunological risk factors. PSC can present at any age and is characterised by a long subclinical asymptomatic period in many cases[1]. The aetiology of the disease is unknown. PSC is closely associated with inflammatory bowel disease (IBD) most commonly ulcerative colitis (UC)[1,2]. A role for perinatal events in the aetiology of IBD has been suggested. Non-specific exposures such as infections and more specific factors including measles virus infection and vaccination as well as appendectomy have been shown to be associated with later development of IBD[3-6]. There is evidence of genetic susceptibility in both IBD and PSC[7,8] and there is a possibility that some immune profiles inherited by the offspring may increase the risk for both these autoimmune diseases as well as for perinatal events and other complications during pregnancy. Since perinatal events represent an increased risk for IBD[6], some early life events may be a direct risk for PSC. The close association between IBD and PSC has been suggested to be part of the pathogenetic explanation for PSC through the existence of an entero-hepatic circulation of lymphocytes. Some lymphocytes generated in the gut during active IBD may subsequently persist as long-lived memory cells and be activated in the liver resulting in hepatic inflammation and the development of PSC. The molecular basis for this hypothesis is that liver and gut share the same lymphocyte homing receptors[9]. However,

this hypothesis does not explain why only 5% of patients with IBD eventually develop PSC[10] and why PSC can precede IBD. Whether perinatal factors play a role for the development of PSC later in life has not been previously evaluated.

The aim of this study was to assess whether perinatal events, intrauterine or postpartum, are associated with an increased risk of PSC.

MATERIALS AND METHODS

All patients with PSC treated at the five largest University Hospitals in Sweden between 1970 and 1998 were selected for this study (n = 311). Through the unique national registration number assigned to every Swedish citizen we were able to trace birth parish to identify the hospital where delivery occurred. To trace the medical records associated with delivery and pregnancy from these patients, we searched the respective hospital archives. The detailed medical records from 97 patients with PSC were collected. Individuals born abroad or delivered at home by midwives were excluded. As controls, we selected two children of the same sex as the subject, who were delivered at the same hospital immediately after the subjects. The controls had to be alive and living in Sweden at the date of PSC diagnosis for the case. Controls who had died or emigrated before the diagnosis of their matched case were excluded. One hundred and seventy five controls remained for analysis.

The diagnosis of PSC was based on biochemical, histological and cholangiographic features[11]. Onset of PSC was defined as first cholangiogram consistent with the diagnosis. Adult PSC was defined as onset of PSC at 16 years of age or later. A diagnosis of UC was based on a typical history and characteristic endoscopic and histological findings[12]. All 97 patients with PSC had undergone colonoscopy to look for IBD and no endoscopic or histological signs of IBD were found in the non-IBD PSC patients.

For both, subjects and controls, we retrieved information on the age of parents, delivery method, parity and maternal complications including proteinuria, peripheral oedema, hypertension or other diseases during the pregnancy. Other conditions and factors during pregnancy included surgeries, infections, nausea, anemia, varices, constipation, thrombosis or any other disease reported by the patients to the midwife and documented in the patient's record. Information concerning medication during pregnancy, gestational age, birth height and weight, weight of the placenta and medical problems experienced by the mother or child postpartum were also collected. Small and large for gestational age were defined as birth weights two standard deviations below and above normal for the gestational age. All data from the birth records were personally collected by one researcher (U.L). The study was approved by the Ethics committee at Karolinska Institute, Karolinska University Hospital, Huddinge, Sweden.

Statistical analysis

Mean values of continuous measurements were compared in order to describe the characteristics of the case and control groups. To assess associations of the perinatal measures with PSC, conditional multiple logistic regression was used. All investigated parameters were modelled as a series of binary dummy variables. After investigation of univariate relationships, all measures associated with PSC ($P < 0.05$) that were statistically significant were included in multivariate analysis using conditional logistic regression. The final conditional logistic regression model excluded redundant measures co-linear with other significant risk factors.

Table 1 Description of perinatal factors in 97 PSC patients and 175 controls

	Patients with PSC (n = 97)	Controls (n = 175)	P
Age of the mother, yr (mean ± SD)	27.8 ± 5.1	27.4 ± 6.2	NS
Hospital stay days (mean ± SD)	7.9 ± 3.2	8.0 ± 4.7	NS
Birth weight in grams (mean ± SD)	3501 ± 537	3557 ± 480	NS
Birth length in cm (mean ± SD)	51.0 ± 2.2	51.0 ± 1.9	NS
Ponderal index (Weight/height^2)	2.6 ± 0.3	2.7 ± 0.3	NS
Vaginal delivery, n (%)	90 (93%)	166 (95%)	NS
Jaundice post partum, n (%)	7 (7%)	14 (8%)	NS
Breastfeeding at discharge from hospital, n (%)[1]	94 (97%)	170 (97%)	NS

[1]Missing data in 2 patients.

RESULTS

The mean age at PSC diagnosis in the 97 patients was 40 ± 12 years (± SD). 78% (76/97) were men, 80% (78/97) had a concomitant diagnosis of IBD. Sixty eight patients had UC, nine were diagnosed with Crohn's disease and one patient had indeterminate colitis.

A description of maternal and perinatal factors in the 97 PSC cases and the 175 controls is given in Table 1. Birth weight (BWT) was divided into five equally sized groups, where BWT 1 represented the lowest birth weight. Compared with the middle category, only the second lowest birth weight group was associated with PSC in a statistically significant manner. There were no statistically significant differences between the rates of maternal infections in subjects and controls. During pregnancy, maternal infections were reported in four cases (pneumonia (n = 1), tuberculosis (n = 1), urinary tract infection (n = 1), rubella in the first trimester (n = 1)). The child born to the rubella-infected mother was female and developed Crohn´s disease before PSC was diagnosed. In the control group, one mother suffered from tuberculosis. None of the mothers were diagnosed with IBD or chronic liver disease. 9.3% of the PSC cases and 8.0% of the controls had postpartum medical issues. The most common problems observed in the children were skin lesions and asphyxia-related problems, in both groups. The summated frequency of

Table 2 Matched odds ratios and 95% confidence intervals for the maternal perinatal risk factors studied in 97 patients with PSC and 175 controls

Risk factor	[1]*n* with PSC (%)	*n* without PSC(%)	OR (95% CI)	*P*
Vaginal bleeding	6 (6.2)	2 (1.1)	5.70 (1.13-28.83)	0.035
Oedema	15 (15.5)	13 (7.4)	2.28 (1.04-5.03)	0.040
Eclampsia	0	1 (0.6)	a	
Albuminuria during pregnancy	10 (11.4)	8 (5.2)	2.33 (0.88-6.15)	0.087
Albuminuria at arrival at the hospital	16 (17.2)	20 (13.1)	1.35 (0.66-2.76)	0.416
Albuminuria postpartum	13 (13.7)	22 (13.3)	1.02 (0.48-2.14)	0.963
Medical problems postpartum-mother	11 (11.3)	22 (12.6)	0.89 (0.41-1.93)	0.765
Medical problems postpartum-child	9 (9.3)	14 (8.0)	1.18 (0.49-2.83)	0.717
Breastfeeding	95 (97.9)	166 (97.1)	1.44 (0.27-7.67)	0.668
BWT 1	23 (23.7)	31 (17.7)	1.44 (0.57-3.65)	0.437
BWT 2	26 (26.8)	28 (16.0)	2.89 (1.17-7.18)	0.022
BWT 3	16 (16.5)	39 (22.3)	Reference	
BWT 4	14 (14.4)	42 (24.0)	0.76 (0.29-2.01)	0.576
BWT 5	18 (18.6)	35 (20.0)	1.44 (0.57-3.63)	0.437
Gestational weeks < 38	8 (8.4)	7 (4.1)	2.20 (0.77-6.29)	0.144
Gestational weeks 38-42	78 (82.1)	150 (87.2)	Reference	
Gestational weeks > 42	9 (9.5)	15 (8.7)	1.16 (0.48-2.76)	0.755
Small for gestational age			1.09 (0.25-4.71)	
Large for gestational age			2.33 (0.61-8.91)	

BWT 1-5, 5ths; 1, lowest ; BWT, body weight. [1]The cases are matched with controls for sex, age and hospital [a]No estimate due to an empty cell.

all postpartum medical problems for mothers of PSC patients was 11.3% and 12.6% for the controls, dominated by bleeding-anaemia in both groups. This difference is not statistically significant.

The matched odds ratios for perinatal factors are shown in Table 2. Both vaginal bleeding and peripheral oedema are statistically significantly associated with PSC. There was also an association of albuminuria during pregnancy with PSC that is not statistically significant. There was no association between jaundice and the risk of PSC. The non-matched odds ratios did not differ notably from the matched odds ratios shown in Table 2 (data not shown).

A multivariate analysis was conducted for the factors associated with a statistically significant increased risk of PSC: vaginal bleeding, peripheral oedema and birth weight. For vaginal bleeding, the adjusted OR (95% CI) is 6.7 (1.3-34.8), 2.4 (1.1-5.3) for peripheral oedema and 2.24 (1.00-5.02) for the second lowest birth weight category. An additional adjustment for gestational age was conducted separately (data not shown) and did not significantly alter the odds ratios for birth weight, vaginal bleeding or peripheral oedema.

DISCUSSION

To our knowledge, this is the first study investigating the associations of perinatal factors with PSC. The rationale for conducting such a study is that the foetal environment may be a contributing factor in the aetiology of some adult conditions such as diabetes, insulin resistance and rheumatoid arthritis[13,14]. In addition, IBD (both CD and UC) is closely associated with PSC and "non-infectious health events" during pregnancy[4,6]. In celiac disease, which is also associated with PSC[15], it has been shown that a low birth weight for gestational age and neonatal infections are associated with later development of celiac disease[16].

We found no associations between PSC and birth length, gestational age, or medical problems for the mother or child, during pregnancy or postpartum. Mothers who had specific infections were identified. Neither individually nor combined were the specific infections associated with PSC risk. However, the number of events is low so detailed analysis was not possible. The association of PSC with birth weight was limited to one part of the birth weight distribution and there was no evidence of a trend. This suggests a chance association that should not be over-interpreted.

Some specific factors, vaginal bleeding during pregnancy and peripheral oedema, proved to be statistically significant in association with higher PSC risk. Interpretation of these data should be cautious. The number of women with vaginal bleeding was small and a clear mechanism to link this event with PSC is not readily apparent. As more women displayed peripheral oedema, its association with PSC is of greater relevance, yet it is a somewhat non-specific symptom, and again, a biologically plausible mechanism linking it with PSC is not obvious. It may be worthwhile to note that peripheral oedema is a symptom of pre-eclampsia; the other important symptoms are hypertension and proteinuria. Only one mother in this study was diagnosed with pre-eclampsia and we could not fully evaluate possible sub-clinical diseases as data were missing for proteinuria in both groups. However, the weak association of proteinuria with the risk for PSC provides further, but limited, evidence that some symptoms of pre-

eclampsia are associated with PSC. If the link between PSC and these maternal symptoms is not due to chance, then they could indicate some inherited characteristics that represent susceptibility to PSC. Alternatively, they might represent foetal exposure, possibly to pro-inflammatory factors, which increase the risk of PSC among susceptible individuals, perhaps through the initiation of an autoimmune process.

Although the detailed aetiology of PSC is unknown, its close association with IBD indicates that these two diseases share important risk factors relevant to exposure or susceptibility.

It is possible that risk factors identified by this study could be intermediates in a causal pathway between IBD and PSC: this could not be investigated here due to the small number of IBD-free subjects. However, it is also possible that the associations are specific to PSC or identify a subset of people with both diagnoses. IBD may indicate greater susceptibility to PSC, but exposure to other risk factors may be required to initiate the pathogenesis of this disease. Given the close association of PSC and IBD, known risks for IBD should be considered.

Bacterial colonisation of the gut is implicated in the aetiology of IBD; this occurs first in early life and the critical stages of early gut colonisation include exposure to bacteria in the birth canal, maternal faecal bacteria and during weaning[17]. Breastfeeding is important for colonic bacterial colonisation[17]. Previous studies investigating the association between breast feeding and development of IBD have shown inconsistent results[18,19]. In the present study, there was a similarly high frequency of breastfeeding among patients and controls (97% in both groups). However, the observation period was short (approximately one week) since breastfeeding was only registered at the time when the mothers were discharged from the hospital and no follow-up was available.

Although the number of subjects included in this study was not large, it includes a high proportion of Swedish PSC patients between the years 1970 and 1998. Differential selection bias is not a concern as subjects and controls were closely matched and the analysis was conditional, such that patients were compared with their matched controls. The controls were selected from the same birth unit (next two consecutive births with the same gender as the subject) and the data was retrieved by the same person. Furthermore, differential information bias is not a determining factor either, as the selection of cases or controls was not influenced by whether perinatal adverse events occurred or not. The general characteristics of the PSC patients in the present study, such as age at onset of PSC, association with IBD and sex distribution, are similar to other studies, suggesting that selection bias among cases is unlikely[1,2].

In summary, no significant associations with PSC were found for gestational age, birth length, breastfeeding, or most medical complications, including infections, during pregnancy for the mothers or postpartum for the children. The associations with vaginal bleeding and some symptoms of pre-eclampsia could be due to chance, but should be considered as putative risk factors by further studies. Overall, our findings do not support the hypothesis of a substantial role for perinatal events in the aetiology of PSC later in life.

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RAPID COMMUNICATION

Loss of disabled-2 expression is an early event in esophageal squamous tumorigenesis

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Abstract

AIM: *Disabled-2* (*DAB2*) is a candidate tumor-suppressor gene identified in ovarian cancer that negatively influences mitogenic signal transduction of growth factors and blocks ras activity. In a recent study, we observed down-regulation of *DAB2* transcripts in ESCCs using cDNA microarrays. In the present study, we aimed to determine the clinical significance of loss of DAB2 protein in esophageal tumorigenesis, hypothesizing that *DAB2* promoter hypermethylation-mediated gene silencing may account for loss of the protein.

METHODS: DAB2 expression was analyzed by immunohistochemistry in 50 primary esophageal squamous cell carcinomas (ESCCs), 30 distinct hyperplasia, 15 dysplasia and 10 non-malignant esophageal tissues. To determine whether promoter hypermethylation contributes to loss of DAB2 expression in ESCCs, methylation status of *DAB2* promoter was analyzed in DAB2 immuno-negative tumors using methylation-specific PCR.

RESULTS: Loss of DAB2 protein was observed in 5/30 (17%) hyperplasia, 10/15 (67%) dysplasia and 34/50 (68%) ESCCs. Significant loss of DAB2 protein was observed from esophageal normal mucosa to hyperplasia, dysplasia and invasive cancer (P_{trend} < 0.001). Promoter hypermethylation of *DAB2* was observed in 2 of 10 (20%) DAB2 immuno-negative ESCCs.

CONCLUSION: Loss of DAB2 protein expression occurs in early pre-neoplastic stages of development of esophageal cancer and is sustained down the tumorigenic pathway. Infrequent *DAB2* promoter methylation in ESCCs suggests that epigenetic gene silencing is only one of the mechanisms causing loss of DAB2 expression in ESCCs.

Key words: *Disabled-2*; DOC-2; Esophageal cancer; Promoter hypermethylation; Dysplasia

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INTRODUCTION

Esophageal cancer is the most aggressive gastrointestinal malignancy ranking as the 6th most common cancer among males and 9th most common cancer among females globally[1]. Squamous cell carcinoma is the predominant histological subtype of esophageal cancer, characterized by high mortality rate and strong association with dietary habits and life style in India[2-4]. It is the 2nd most common cancer among males and 4th most common cancer among females in India[5]. Despite advances in multimodality therapy, due to late stage of diagnosis and poor efficacy of treatment, the prognosis for patients with ESCC still remains poor with an average 5-year survival of < 10% globally[6,7] and < 12% in India[8,9].

DOC-2/DAB2 (differentially expressed in ovarian carcinoma- 2/disabled-2) is a putative tumor suppressor gene that encodes a 96-ku mitogen-responsive phosphoprotein involved in signal transduction[10-15]. It inhibits mitogenic stimulation via the Ras pathway by binding to Grb2[13,14]. DAB2 has been shown to act as a negative regulator of c-Src in normal prostatic epithelium and cancer[16]. This interaction causes inactivation of Erk and Akt proteins critical for proliferation and survival of prostate cancer cells[16,17]. DAB2 is found in association with transforming growth factor-β (TGF-β) type Ⅰ and Ⅱ receptors, while directly binding to the TGF-β signaling intermediates Smad2 and Smad3 through the PID domain[18]. DAB2 plays an important regulatory role in cellular differentiation and induction of differentiation in the absence of DAB2 expression commits the cell to apoptosis[19]. DAB2 functions as a negative regulator of canonical Wnt signaling by stabilizing the beta-catenin

degradation complex[20]. Recently, treatment of mouse F9 embryonic carcinoma cells with glycosylceramide synthase inhibitors has been shown to result in depletion of gangliosides and delayed expression of DAB2, suggesting their involvement in F9 cell differentiation[21]. The aberrant expression of DAB2 has been reported in tumors, such as ovarian, prostate, choriocarcinoma, and breast[11,22-26]. Histologically, elevated levels of DAB2 are associated with an enriched basal cell compartment, a progenitor cell for glandular epithelium and may be involved in the homeostasis of rat prostate regeneration[23]. In addition, stable expression of DAB2 in cancer cell line has been shown to significantly reduce its *in vitro* growth rate, concomitant with an increase in cells in G_1 and decrease in anchorage-independent growth on soft agar[11,22,23]. Therefore, DAB2 appears to be a potent negative regulator of cancer cell growth.

In a recent study, we observed down-regulation of *DAB2* transcripts in ESCCs using cDNA microarrays (data not shown). To our knowledge, the clinical significance of down-regulation of DAB2 in ESCC remains to be determined. In the present study, we analyzed DAB2 protein expression in different stages of development of esophageal cancer viz., primary ESCC and paired non-malignant normal, hyperplasia and dysplasia. Loss of DAB2 protein was observed in high proportion of ESCCs and dysplasia. Therefore, we hypothesized epigenetic silencing of *DAB2* gene in ESCCs. To test this hypothesis, the methylation status of the putative promoter (exon 1) of *DAB2* was analyzed using methylation-specific PCR in ESCC tissues that showed loss of DAB2 protein.

MATERIALS AND METHODS

Tissue samples

The study was approved by Institutional Human Ethics Committee and informed consent was obtained from the patients prior to enrolment in the study. The tissue samples used in this study were collected from Department of Gastrointestinal Surgery, All India Institute of Medical Sciences, New Delhi, India. All the samples were histologically confirmed to be either ESCCs, esophageal hyperplasia, dysplasia or non-malignant tissues by the pathologist (SDG). The samples included 50 histologically confirmed ESCCs, 10 non-malignant esophageal mucosa, 30 hyperplasia and 15 dysplasia.

Immunohistochemistry

Immunohistochemical analysis of DAB2 protein was carried out in paraffin-embedded tissue sections (5 μm thickness). Briefly, the sections were deparaffinized in xylene, hydrated and incubated with 30 mL/L H_2O_2 in methanol for 5 min to inactivate the endogenous peroxidase. Slides were washed with Tris-buffered saline (TBS, 0.1 mol/L, pH7.4) and heated for 15 min at 100℃ in 10 mmol/L sodium citrate buffer (pH 6.0). Thereafter, sections were incubated with anti-disabled-2 goat polyclonal antibody (C-20, dilution 1:50, Santa Cruz Biotechnology Inc., Santacruz, CA) at 4℃ overnight in humidified chamber. Sections were incubated with biotinylated anti-mouse antiserum with horseradish peroxidase streptavidin conjugate (DAKO Labs, Glostrup, Denmark). After every incubation step, slides were washed with TBS thrice and color was developed using 3,3' -diaminobenzidine hydrochloride (DAB). Sections were counterstained with Mayer's hematoxylin, and mounted with DPX mountant for evaluation. Normal ovary tissue sections were taken as positive control for DAB2 and in the negative control primary antibody was replaced by isotype-specific IgG (data not shown).

Bisulfide modification

Genomic DNA from tissues was isolated by phenol-chloroform method. Genomic DNA was treated with sodium bisulfite (Sigma-Aldrich, Bangalore, India) as previously described[27]. Briefly, 1 μg of DNA was denatured with 0.2 mol/L NaOH for 10 min at 37℃. Thirty microliters of 10 mmol/L hydroquinone (Sigma Aldrich) and 520 μL of 3 mol/L sodium bisulfite (pH 5.0) were added, followed by incubation at 50℃ for 16 h. The modified DNA was purified using Wizard DNA purification columns (Promega, Madison, WI). The purified DNA was desulphonated with NaOH and precipitated with absolute ethanol in the presence of glycogen and ammonium acetate. DNA was resuspended in 20 μL of 1 mmol/L Tris (pH 8.0) and used for PCR amplification.

Methylation-specific PCR

Bisulfite-treated genomic DNA was amplified with either a methylation-specific or unmethylation-specific primer set for 35 cycles at 95℃ for 5 min (hot started by adding Taq polymerase), followed by cycling with denaturation at 95℃ for 30 s, primers annealing at 60℃ for 30 s, and extension at 72℃ for 1 min, as well as a final extension step at 72℃ for 5 min. Methylation-specific primers span 6 CpG dinucleotides numbered 19-21 (forward) and 35-37 (reverse) of *DAB2* exon1 (Gene accession No. AF218839). Similarly, unmethylation-specific primers span 8 CpG dinucleotides numbered 19-22 (forward) and 35-37 (reverse). The methylation-specific primers were designed using 5'-TATTTTTCGTCGGGAGTGGTCGC-3' as the forward primer and 5'-ACTAACTATTACCTCCGTAAA ACG-3' as the reverse primer. The unmethylation-specific primers for site S1 were designed using 5'-GAATTATATT TTTTGTTGGGAGTGGTTGT-3' as the forward primer and 5'-CCAACTAACTATTACCTCCATAAAACA -3' as the reverse. These primer sequences were reported by Akiyama *et al*[28].

RESULTS

Immunohistochemical analysis of DAB2

Immunohistochemical analysis was carried out to determine the expression of DAB2 protein in different stages of esophageal tumorigenesis. Table 1 summarizes the clinicopathological parameters of ESCC patients and expression status of DAB2 protein in the tumors. Strong cytoplasmic staining of DAB2 was observed in epithelial cells of all the 10 non-malignant (histologically normal) esophageal mucosa (Figure 1A). No detectable expression of DAB2 protein was observed in 5/30 (17%) hyperplasia

Table 1 Correlation of DAB2 protein expression with clinicopathological parameters of ESCC patients

Parameters	Total cases	DAB2	
	n	Positive *n* (%)	Negative *n* (%)
ESCC	50	16 (32)	34 (68)
Age			
⩽ 40 yr	14	4 (28)	10 (72)
⩾ 40 yr	36	12 (33)	24 (67)
Gender			
Male	30	11 (37)	19 (63)
Female	20	5 (25)	15 (75)
Tumor Stage			
T2	3	1	2
T3	36	11 (31)	25 (69)
T4	11	4 (36)	7 (64)
Hp Grading			
WDSCC	8	2	6
MDSCC	37	11 (30)	26 (70)
PDSCC	5	3 (60)	2 (40)
Nodal stage			
Node -ve	23	8 (35)	15 (65)
Node +ve	27	8 (30)	19 (70)
Normal	10	10	
Hyperplasia	30	25 (83)	5
Dysplasia	15	5	10 (67)

(Figure 1B), 10/15 (67%) dysplasia (Figure 1C) and 34/50 (68%) ESCCs (Figure 1D). Only 32% of ESCCs showed weak to moderate DAB2 staining (Figure 1E). Significant loss of DAB2 protein expression was observed from esophageal normal mucosa to hyperplasia, dysplasia and SCC ($P_{trend} < 0.001$). Significantly higher proportion of dysplastic tissues showed loss of DAB2 expression in comparison with hyperplasia ($P = 0.002$; OR = 9.998; 90% CI = 2.368-42.210).

DAB2 exon1 methylation in ESCCs

To determine the possibility of promoter methylation-mediated gene silencing of *DAB2*, methylation status of putative promoter in exon1 of *DAB2* gene was analyzed in 10 DAB2 immuno-negative ESCC tissues. Burkitt's lymphoma cell line Raji was used as a positive control for hypermethylation of *DAB2* gene, as reported by Akiyama *et al*[28]. As shown in Figure 2, 8 of 10 (80%) ESCCs showed signal (PCR amplicon) for the unmethylated *DAB2* alleles, while 2 cases showed the presence of both unmethylated and methylated PCR amplicon, suggesting that *DAB2* promoter hypermethylation may account for loss of DAB2 protein in these two tumors.

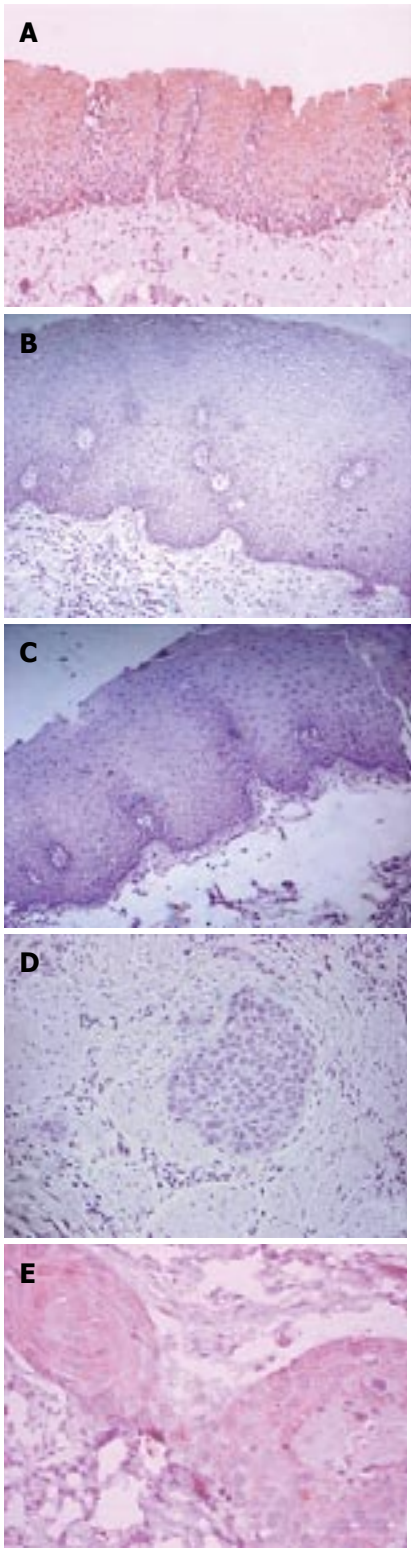

Figure 1 Immunohistochemical analysis of DAB2 in esophageal squamous cell carcinogenesis. **A:** Cytoplasmic expression of DAB2 in non-malignant normal esophageal mucosa; **B:** Hyperplastic esophageal mucosa showing no detectable expression of DAB2 protein; **C:** Dysplastic esophageal mucosa showing loss of DAB2 protein expression; **D:** ESCC section showing loss of DAB2 protein expression; and **E:** ESCC showing faint cytoplasmic DAB2 protein expression (**A-D**: Original magnification x 100; and **E**: Original magnification x 200).

DISCUSSION

In this study, we demonstrated, probably for the first time, significant loss of DAB2 protein in different stages of development and progression of ESCC, from normal to hyperplasia, dysplasia and invasive cancer ($P_{trend} < 0.001$). Strong cytoplasmic expression of DAB2 protein was observed in all the 10 histologically non-malignant esophageal tissues. Loss of DAB2 protein was observed in preneoplastic stages, as early as in hyperplasia (in 5 of 30 hyperplastic tissues analyzed), suggesting that deregulation of DAB2 expression is likely to be an early event in esophageal tumorigenesis. Interestingly, loss of DAB2 protein was observed in significantly higher proportion of dysplastic tissues (10/15 cases) in comparison with hyperplasia ($P = 0.002$), indicating a critical impact of loss of this protein in evolution of dysplasia. Furthermore, loss of DAB2 in 34/50 (68%) of ESCCs observed in this study suggests that down-regulation of DAB2 protein is sustained down the tumorigenic pathway. To our

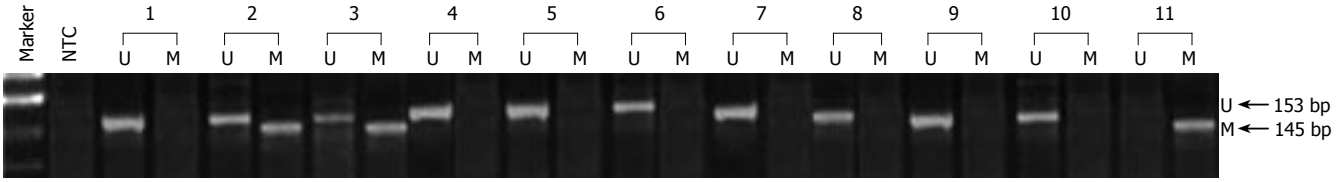

Figure 2 DAB2 exon-1 promoter hypermethylation. Methylation-specific PCR was done to determine the possibility of promoter methylation-mediated gene silencing of *DAB2*. Eight of 10 ESCCs showed signal (PCR amplicon) for the unmethylated *DAB2* alleles (samples 1, 4-10), while 2 cases showed the presence of both unmethylated and methylated PCR amplicon (samples 2 and 3). Burkitt's lymphoma cell line Raji was used as a positive control for hypermethylation of *DAB2* gene (sample 11). In each sample, lane U represents the unmethylated product, while M represents the methylated product, lane NTC refers to the no template control.

knowledge, the clinical relevance of DAB2 in primary human esophageal tumors remains to be determined. Therefore, our study is important in demonstrating the clinical significance of aberrant DAB2 expression in primary ESCCs.

The physical interaction of epithelial cells with the basement membrane ensures correct positioning and acts as a survival factor for epithelial cells. Cells that detach from the basement membrane often undergo apoptosis[29,30]. In tumors, this positional control is absent, resulting in disorganized cell proliferation[26]. Inactivation of a gene(s) controlling epithelial cell positioning may be a step in tumorigenicity. One of these genes is *DAB2*, which functions in cell positioning control and loss of DAB2 protein has been suggested to contribute to the basement membrane-independent, disorganized proliferation of tumor cells[26]. DAB2 expression in breast cancer cells resulted in sensitivity to suspension-induced cell death (anoikis)[31]. Loss of DAB2 expression and the loss of collagen IV and laminin-containing basement membrane are two critical events associated with morphologic dysplastic changes of the ovarian surface epithelium as a step in tumorigenicity[32,33].

Basement membrane (BM) can regulate differentiation, proliferation and polarity of esophageal epithelium and its integrity is important for carcinogenesis. Studies aimed to explore the effect of the BM changes induced by chronic inflammation on esophageal carcinogenesis have suggested that BM changes with aberrant proliferation of esophageal epithelia[34]. The most salient finding of our study is the significant loss of DAB2 protein expression in pre-neoplastic lesions, such as dysplasia in comparison with non-malignant esophageal epithelium and hyperplasia ($P = 0.002$). Based on the studies in ovarian cancer and transitional cell carcinoma and our observations in esophageal dysplasia and ESCC, we hypothesize that down-regulation of DAB2 protein in dysplasia may be an important step in loss of epithelial cell positioning, aberrant proliferation and tumorigenicity. Therefore, loss of DAB2 protein may serve as a candidate molecular marker for pre-neoplastic lesions.

Akiyama *et al*[28] showed epigenetic silencing of GATA-4 and GATA-5 but not GATA-6 transcription factor genes and their potential downstream anti-tumor target genes in colorectal and gastric cancer. GATA6 and histone deacetylase inhibitor synergistically induce *DAB2* gene expression in transitional cell carcinoma (TCC) cell lines[35]. Histone acetylation status associated with the 5' upstream regulatory sequence of *DAB2* gene is one of the key determinants of its activity. GATA6 can specifically induce *DAB2* promoter activity. Increased histone acetylation and the presence of GATA6 have a synergistic effect on *DAB2* promoter activity which results in elevation of DAB2 protein expression. Although the underlying mechanism leading to high GATA6 and/or acetyl H3 levels in TCC cell lines is still unclear, it is likely that enzymes responsible for epigenetic regulation, such as histone modification or DNA methylation, could play a role. Therefore, we analyzed the methylation status of *DAB2* gene in esophageal tumors that showed loss of DAB2 protein. Methylation-specific PCR showed methylation of *DAB2* promoter in 2 of the 10 DAB2 immuno-negative ESCCs analyzed. These findings suggest that epigenetic silencing of *DAB2* is infrequent in ESCCs and accounts for down-regulation of the protein in only a subset of esophageal tumors. Thus, there is a need to investigate other mechanisms that may be responsible for loss of DAB2 protein in ESCCs harboring the unmethylated *DAB2* promoters. A parallel study on DAB2 protein expression and promoter methylation in our laboratory showed similar discordance between loss of protein expression and epigenetic silencing of the gene in breast cancer. *In silico* analysis suggested that post-transcriptional, micro RNA-mediated targeting of *DAB2* mRNA may be another mechanism for gene silencing (unpublished data of Bagadi SAR *et al*). It will be worthwhile to determine if micro RNA is involved in targeting of *DAB2* mRNA accounting for loss of DAB2 protein in ESCCs as well.

In conclusion, our data suggest that loss of DAB2 protein occurs in early pre-neoplastic stages and is sustained down the tumorigenic pathway of esophageal squamous cell carcinogenesis, underscoring its potential as a candidate molecular marker for pre-neoplastic lesions. Furthermore, *DAB2* exon-1 promoter hypermethylation is an infrequent event in ESCCs.

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RAPID COMMUNICATION

Inhibition of hepatitis B virus expression and replication by RNA interference in HepG2.2.15

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Abstract

AIM: To observe the inhibition of hepatitis B virus replication and expression by transfecting vector-based small interference RNA (siRNA) pGenesil-HBV X targeting HBV X gene region into HepG2.2.15 cells.

METHODS: pGenesil-HBV X was constructed and transfected into HepG2.2.15 cells *via* lipofection. HBV antigen secretion was determined 24, 48, and 72 h after transfection by time-resolved immunofluorometric assays (TRFIA). HBV replication was examined by fluorescence quantitative PCR, and the expression of cytoplasmic viral proteins was determined by immunohistochemistry.

RESULTS: The secretion of HBsAg and HBeAg into the supernatant was found to be inhibited by 28.5% and 32.2% ($P < 0.01$), and by 38.67% ($P < 0.05$) and 42.86% ($P < 0.01$) at 48 h and 72 h after pGenesil-HBV X transfection, respectively. Immunohistochemical staining for cytoplasmic HBsAg showed a similar decline in HepG2.2.15 cells 48 h after transfection. The number of HBV genomes within culture supernatants was also significantly decreased 48 h and 72 h post-transfection as quantified by fluorescence PCR ($P < 0.05$).

CONCLUSION: In HepG2.2.15 cells, HBV replication and expression is inhibited by vector-based siRNA pGenesil-HBV X targeting the HBV X coding region.

Key words: Hepatitis B virus; RNA interference; Plasmid vector; HepG2.2.15

Zhao ZF, Yang H, Han DW, Zhao LF, Zhang GY, Zhang Y, Liu MS. Inhibition of hepatitis B virus expression and replication by RNA interference in HepG2.2.15. *World J Gastroenterol* 2006; 12(37): 6046-6049

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INTRODUCTION

Hepatitis B is a severe infectious disease threatening peoples' health all over the world. There is still no efficient therapy to control HBV persistent replication, which may lead to the development of liver cirrhosis and hepatocellualar carcinoma (HCC)[1]. RNA interference (RNAi) is a highly specific and effective mechanism of post-transcriptional gene silencing mediated by double-stranded RNA of 21-23 nt in size. Several researches have suggested that RNAi could provide a new therapeutic strategy against chronic HBV infection[2-6]. In the present study, a plasmid leading to the expression of small interfering RNA (siRNA) that targets the HBV X gene was transfected into HepG2.2.15 cells, and HBV DNA replication as well as HBV antigen expression and secretion were monitored.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco BRL, USA. Metafectene transfection reagent was purchased from Germany Biontes. Diagnostic kits for HBsAg and HBeAg (time-resolved immunofluorometric assay) were obtained from Suzhou Xinbo Biotechnology Corporation. Mouse monoclonal antibody directed against human HBsAg and rabbit anti-mouse horseradish peroxidase (HRP)–IgG were purchased from Beijing Zhongshan Golden Bridge Biotechnology Corporation. HepG2.2.15 cells were obtained from the Institute of Infectious Disease, Beijing University Medicine School.

Plasmid construction

pGenesil, containing human U6 promoter, was used to generate a series of siRNA expression vectors by inserting annealed oligonucleotides between *BamH*I and *Hind*Ⅲ sites. The oligonucleotides 5'-GAT CCG GTC TTA CAT AAG AGG ACT TTC AAG ACG AGT CCT CTT ATG TAA GAC CTT TTT TGT CGA CA-3' (sense) and 3'

Table 1 Effect of pGenesil-siHBV X on HBsAg and HBeAg expression transfected HepG2.2.15 cells

Group	HBsAg (μg/L)			HBeAg (Ncu/mL)		
	24 h	48 h	72 h	24 h	48 h	72 h
Untreated	7.13 ± 0.20	14.43 ± 0.56	22.50 ± 2.14	0.43 ± 0.02	0.75 ± 0.06	1.12 ± 0.10
Metafectene	6.80 ± 0.17	13.57 ± 0.88	21.84 ± 0.91	0.48 ± 0.02	0.69 ± 0.08	1.03 ± 0.06
pGenesil	6.57 ± 0.40	14.30 ± 0.47	24.15 ± 2.24	0.45 ± 0.06	0.64 ± 0.05	1.00 ± 0.06
PGenesil-HK	6.47 ± 0.20	13.63 ± 0.64	21.84 ± 0.51	0.46 ± 0.05	0.69 ± 0.07	0.97 ± 0.08
PGenesil-AFP	6.33 ± 0.35	13.70 ± 0.73	23.11 ± 1.25	0.47 ± 0.05	0.76 ± 0.08	1.10 ± 0.12
PGenesil-HBVX	5.97 ± 0.13	10.25 ± 0.32[b]	15.26 ± 0.88[b]	0.43 ± 0.02	0.46 ± 0.01[c]	0.64 ± 0.04[c]

[b]$P < 0.01$ *vs* untreated control and other controls; [c]$P < 0.05$ *vs* untreated control and other controls.

-GCC AGA ATG TAT TCT CCT GAA AGT TCT GCT CAG GAG AAT ACA TTC TGG AAA AAA CAG CTG TTC GA-5' (antisense) were used for the construction of pGenesil-HBV X targeting HBV X (N 1649 to 1667)[7]; 5'-GAT CCG CAT TG G CAA AGC GAA GCT TTC AAG ACG AGC TTC GCT TTG CCA ATG CTT TTT TGT CGA CA-3' (sense) and 3'-GCG TAA CCG TTT CGC TTC GAA AGT TCT GCT CGA AGC GAA ACG GTT ACG AAA AAA CAG CTG TTC GA-5' (antisense) for a control vector targeting the α-fetoprotein (AFP) gene (N 1275 to 1293); and 5'-GAT CCG ACT TCA TAA GGC GCA TGC TTC AAG ACG GCA TGC GCC TTA TGA AGT CTT TTT TGT CGA CA-3' (sense) and 3' -GCT GAA GTA TTC CGC GTA CGA AGT TCT GCC GTA CGC GGA ATA CTT CAG AAA AAA CAG CTG TTC GA-5' (antisense) for the control vector pGenesil-HK producing a random sequence of siRNA.

Cell culture and transfection

HepG2.2.15 cells were maintained in DMEM supplemented with 100 mL/L fetal calf serum (FCS), 100 IU/mL penicillin, 100 mg/L streptomycin and 2 mmol/L L-glutamine at 37℃ in an atmosphere of 50 mL/L CO_2. The cells were plated in 6-well plates which had been placed sterile cover slips (1×10^6 cells per well). Transfection was performed at about 70% confluence with pGenesil and Metafectene lipofection reagent complex at a ratio of 8 μL:2.5 μg. Under these conditions we obtained a 55% transfection efficiency (data not shown).

Quantitative assay of HBsAg and HBeAg

The levels of HBsAg and HBeAg in culture supernatants were measured at 24, 48 and 72 h after transfection by using time-resolved immunofluorometric assay kits (TRFIA) according to the supplier's instructions.

Assay of HBV DNA replication

HepG2.2.15 cells were harvested at 24, 48, and 72 h post-transfection. Forty μL of the supernatant were mixed with an equal volume of the DNA extractant. Samples were boiled for 10 min and then centrifuged at 10000 × *g* for 5 min. Two μL of the samples were transferred into PCR reaction tubes. PCR cycling parameters consisted of denaturation at 93℃ for 2 min, followed by 93℃ for 45 s; 55℃ for 60 s × 10 cycles and then 93℃ for 30 s; 55℃ for 45 s × 30 cycles.

Immunocytochemistry of HBsAg

To examine whether the effects of pGenesil-HBV X on HBsAg production were uniform with the culture media, cytoplasmic HBsAg was visualized by indirect immunocytochemistry 48 h post-transfection. pGenesil-HBV X transfected and control cells were washed with PBS, fixed in 900 mL/L ethanol for 10 min at room temperature and then washed with PBS. The fixed cells were permeabilized with 5 mL/L Triton X-100 in PBS for 15 min at 37℃ and washed with PBS. To inhibit endogenous peroxidase, cells were exposed to 3 mL/L hydrogen peroxide for 10 min at 25℃. After washed with PBS, cells were incubated with mouse monoclonal anti-HBsAg antibody for 2 h at 37℃ and subsequently with rabbit anti-mouse IgG conjugated horseradish-peroxidase for 30 min at 37℃. Cells were visualized with 3, 3'-diaminobenzidine tetrahydrochloride substrate and examined by light microscopy.

Statistical analysis

All statistical analysis were performed using the Microsoft SPSS 12.0 software. The graphs represented in mean ± SD and compared using unpaired t-test. $P < 0.05$ was regarded as a significant difference.

RESULTS

pGenesil-HBV X inhibited HBsAg and HBeAg secretion in cultured HepG2.2.15

HBsAg and HBeAg concentrations were measured in cell culture supernatants of pGenesil-HBV X treated and control cells 24, 48, and 72 h post-transfection by using TRFIA (Table 1). At 24 h in the culture media, there was no significant difference between pGenesil-HBV X treated cells and other controls (untreated control, pGenesil-AFP control , pGenesil-HK control, pGenesil alone and Metafectene alone) ($P > 0.05$), while HBsAg was inhibited at 48 and 72 h by 28.47% and 32.16% ($P < 0.01$). HBeAg was reduced at 48 and 72 h post-transfection ($P < 0.05$) by 38.7% and 42.9% in the media of pGenesil-HBV X treated cells compared to the controls.

Inhibited HBV DNA replication in cultured HepG2.2.15

Levels of HBV DNA were examined by fluorescence quantitative PCR. This assay can detect HBV DNA in the range of 10^3 to 10^8 copies. The results revealed a

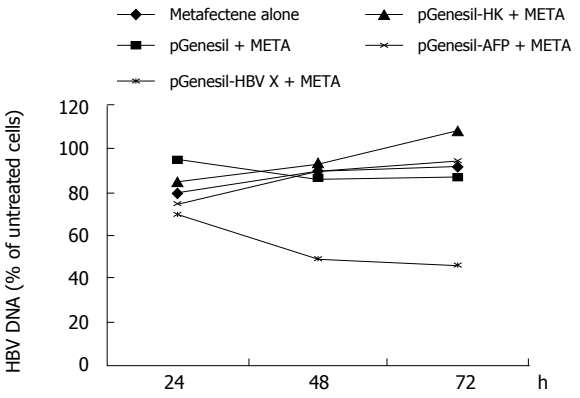

Figure 1 Inhibition of pGenesil-HBV X on HBV DNA in HepG2.2.15.

significant decrease in DNA replication when pGenesil-HBV X treated cells were compared to untreated cells. The number of HBV DNA copies in pGenesil-HBV X treated cells was found to be reduced by 44.9% and 45.9% at 48 and 72 h after transfection, respectively ($P < 0.05$), while the other controls showed no significant difference to the untreated cultures at any time point (Figure 1).

The effects of pGenesil-HBV X on intracellular HBsAg in HepG2.2.15

The effects of pGenesil-HBV X on intracellular HBsAg were visualized by immunocytochemistry 48 h after transfection. Intracellular HBsAg is localized in the cytoplasm of HepG2.2.15 cells at normal. In cells treated with pGenesil-HBV X, HBsAg was either decreased or non-detectable. In contrast, cells of other controls were obviously stained (Figure 2).

DISCUSSION

RNAi is a mechanism of post-transcriptional gene silencing mediated by double-stranded RNA of 21-23 nt in length. RNAi has been applied as a highly specific and efficient tool to interfere with viral replication as it has been shown for HIV[8-12], hepatitis C virus[13-16], myetitis virus[17], gamma herpesvirus[18] or influenza virus[19]. HBV DNA replication requires reverse transcription to form a pregenomic RNA that is similar to reverse-transcription virus. 3.2kb pregenomic mRNA is not only translated into HBV proteins including HBeAg, HBcAg and HBV DNAP, but it is also a template for the synthesis of viral DNA to continue replication. Furthermore, all four transcripts include the HBV X protein coding region. Selecting a conserved sequence in the X gene region as a target, we expect to inhibit the expression of HBV antigens and the replication of HBV DNA. To set up an in vitro model which is more stable and more similar to natural condition of viral infection is a base of experiment and is a key prerequisite to evaluate the efficiency of anti-virus therapy. Previous studies were carried out by co-transfecting siRNA or siRNA expression vector and a plasmid cloned with full-length HBV DNA or HBV target region into cells[7,20,21]. It is obvious that cells transfected with siRNA or siRNA expression vectors must get HBV expression plasmid at same time, which is better to observe and evaluate the specificity and efficiency by siRNA. But only selecting out the cells co-transtected successfully for research does not coincide with natural conditions of viral infection and will not reflect the exact effects of siRNA on HBV target gene.

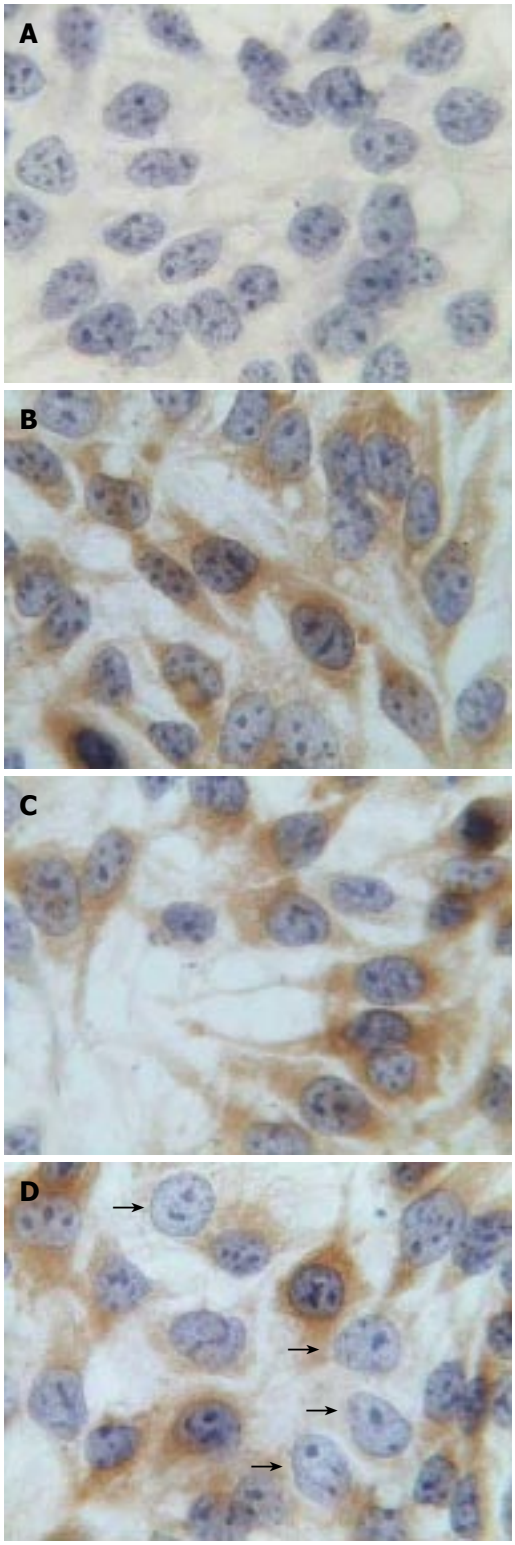

Figure 2 Immunocytochemistry for intracellular HBsAg in HepG2.2.15 (× 400). **A**: Without monoclonal antibody against HBsAg; **B**: pGenesil-HK treated cells; **C**: pGenesil-AFP treated cells; **D**: pGenesil-HBV X treated cells. Arrows show the cells were transfected and HBsAg expression was suppressed.

The HepG2.2.15 cell line, a derivative of the human HepG2 hepatoma cell line that has been stably transformed with a head-to-tail dimer of HBV DNA[22], was chosen as a model because it produces HBV infectious particles constitutively and expresses HBV antigens stably. To transfected siRNA expression vector into HepG2.2.15 cells, and culturing transfected cells and untransfected cells under one system, we could simulate the nature condition that virus still replicate and express constitutively in untransfected cells. As a result, we found that HBsAg and HBeAg in the supernatant were inhibited by 28.5% and 38.7% at 48 h, and decreased by 32.2% and 42.9% 72 h post-tranfection with pGenesil-HBV X against HBV X. Levels of HBV DNA were also found to be reduced. Moreover, immunocytochemistry revealed that the amount of intracellular HBsAg parallels the decline in HBV serum markers in cultures treated with pGenesil-HBV X. Controls (including untreated cells and treated with Metafectent reagent alone, pGenesil plasmid alone, pGenesil-AFP or pGenesil-HK expressing random siRNA) failed to reduce HBV expression and replication. At the premises of targeting same sequence in HBV X region, the efficiency we got was lower than that of Shlomai[7] who used a co-transfection approach to Huh-7 cells. At present, there isn' t any reagent that achieves 100% transfection efficiency neither to cells nor to animals. Selecting HepG2.2.15 cell line as a model is better for us to evaluate the effects of siRNA in consideration of the efficiency of transfection, and thus it will be valuable for us to evaluate the effects of siRNA on clinical application research in the future.

By measuring the levels of AFP which is constitutively secreted by HepG2.2.15 cells no change was found between pGenesil-HBV X treated cells and other controls except pGenesil-AFP treated cells. This result shows that pGenesil-HBV X specifically inhibits HBV gene expression.

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RAPID COMMUNICATION

Expression of matrix metalloproteinase-1 and tissue inhibitor of metalloproteinase-1 in ulcerative colitis

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Abstract

AIM: To examine the expression of metalloproteinase-1 (MMP-1) and tissue inhibitor of metalloproteinase-1 (TIMP-1) in the colonic mucosa of patients with ulcerative colitis (UC).

METHODS: Reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry were used to study the expression of MMP-1 and TIMP-1 at both mRNA and protein levels in patients with UC and controls. The relationship between MMP-1 mRNA, TIMP-1 mRNA, MMP-1 mRNA/TIMP-1 mRNA ratio and the severity of clinical symptoms of the patients with UC were also analyzed.

RESULTS: The expression of MMP-1 mRNA and TIMP-1 mRNA in the ulcerated and inflamed colonic mucosa was significantly higher than that in the non-inflamed colonic mucosa ($P < 0.001$), but there was no statistically significant difference in the non-inflamed colonic mucosa of UC patients and normal controls ($P > 0.05$). The mRNA expression of MMP-1 and TIMP-1 in ulcerated colonic mucosa of UC patients was increased by 80-fold and 2.2-fold, respectively when compared with the normal controls. In the inflamed colonic mucosa, the increase was 30-fold and 1.6-fold, respectively. Immunohistochemical analysis showed that among the ulcerated, inflamed, and non-inflamed colonic mucosae of UC patients and the normal controls, the positive rate of MMP-1 expression was 87%, 87%, 40% and 35% respectively, and the positive rate of TIMP-1 expression was 89%, 89%, 80% and 75%, respectively. Furthermore, the expression of MMP-1 mRNA, TIMP-1 mRNA and the MMP-1 mRNA/TIMP-1 mRNA ratio were correlated with the severity of clinical symptoms ($P <0.05$).

CONCLUSION: Excessive expression of MMP-1 in the diseased colonic mucosa causes excessive hydrolysis of the extracellular matrix (ECM) and ulceration in UC patients. MMP-1 mRNA, TIMP-1 mRNA and MMP-1 mRNA/TIMP-1 mRNA ratio can be used as biomarkers to judge the severity of clinical symptoms in patients with UC. Exogenous TIMP-1 or MMP-1 inhibitor therapy is a novel treatment for patients with UC.

Key words: Matrix metalloproteinase-1; Tissue inhibitor of metalloproteinase-1; Ulcerative colitis; Reverse transcription-polymerase chain reaction; Immunohistochemistry

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INTRODUCTION

Ulcerative colitis (UC) is a chronic, non-specific inflammatory disease of the colonic mucosa with unknown etiology and pathogenesis. Pathologically, it is characterized by ulceration in the mucosal and submucosal areas, and degradation of extracellular matrix (ECM) is one of the major events during this process[1]. Matrix metalloproteinase-1 (MMP-1) produced by cytokine-activated interstitial cells is one of the most important enzymes in degrading ECM, and the activity of MMP-1 is controlled by its natural inhibitor, tissue inhibitor of metalloproteinase (TIMP-1)[2]. Therefore, in this study we measured MMP-1 and TIMP-1 transcripts and their proteins by using reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry to explore their possible role in patients with UC.

MATERIALS AND METHODS

Patients and samples

Thirty patients with UC confirmed by colonoscopy and biopsy were enrolled in the study. Among these patients, 21 were males and 9 were females with their age ranged from 18 to 76 years and averaged 48 years. Samples were taken from the ulcerated, inflamed and non-inflamed areas of the colonic mucosa during colonoscopy. There were 3 patients with pan-colonic lesions, 2 with hemi-colonic lesions, 13 with recto-sigmoidal lesions, and 12 with rectal lesions. Based on the clinical manifestations, 2 patients

were classified into severe type, 18 into moderate type, and 10 into mild type. Meanwhile, 20 normal subjects were selected as controls; 14 of them were males and 6 were females with their age ranged from 27 to 65 years and averaged 46 years. Biopsy samples were immediately snap frozen in liquid nitrogen and stored at -80℃ for RT-PCR. Separate biopsy samples were fixed in formalin and embedded in paraffin for immunohistochemistry.

Total RNA extraction

Total RNA was extracted from the frozen samples using the RNA isolation kit (Invitrogen Company) following the manufacturer's instructions. Five μL of the extracted RNA was run on 1% agarose gel electrophoresis to identify the extracted products.

RT-PCR for MMP-1 and TIMP-1

RT-PCR was performed using the TaKaRa RNA PCR kit 3.0 (AMV) (supplied by Dalian Baosheng Biotechnology Company) following the manufacturer's instructions. Primer sequences[3] used were as follows: MMP-1: sense: 5'-ATGCGAACAAATCCCTTCTACC-3', antisense: 5'-TTTCCTCAGAAAGAGCAGCATCG-3'; TIMP-1: sense: 5'-GGACACCAGAAGTCAACCAGCC-3', antisense: 5'-CGTCCACAAGCAATGAGTCC-3'. Primers forβ-actin were used as the internal control: sense: 5'-CCTTCCTGGGCATGGAGTCCTG-3', antisense: 5'-GGAGCAATGATCTTGATCTTC-3'. Reverse transcription was carried out at 30℃ for 10 min, at 42℃ for 30 min, at 99℃ for 5 min, and at 5℃ for 5 min. PCR was performed as follows: initial denaturation at 94℃ for 2 min, 35 amplification cycles at 94℃ for 30 s, at 53℃ for 30 s, at 72℃ for 1 min, extension at 72℃ for 10 min. Five μL PCR product was run on 2% agarose gel electrophoresis.

Immunohistochemistry

Sample sections were washed 3 times with PBS, 3 min each time after initial treatment. Primary antibodies, rabbit anti-human MMP-1 polyclonal antibody and TIMP-1 monoclonal antibody (Beijing Zhongshan Biology Company) were added and incubated at room temperature for 1.5 h, washed again and incubated with peroxidase-conjugated secondary antibody for 15 min and washed again. A brown product was developed in diaminobenzidine (DAB) for 10 min.

Result determination and statistical analysis

Bio-imaging system (PALL Company, USA) was employed to analyze the density of the bands of PCR products. MMP-1 mRNA and TIMP-1 mRNA were semi-quantitatively expressed by the ratios between MMP-1, TIMP-1 and β-actin OD values. All values were expressed as mean ± SD.

Results of immunohistochemistry were scored according to the degree of staining and percentage of positive cells as no staining: 0 point, mild staining: 1 point, moderate staining: 2 points, heavy staining: 3 points; positive cells ≤ 5%: 0 point, 6%-30%: 1 point, 31%-70%: 2 points, 71%-100%: 3 points. Final score was determined by combining points obtained from the above two scoring systems: 0-1 point: (-), 2 points: (+), 3-4 points: (++), 5-6 points: (+++).

Student-Neuman-Keuls test was used to compare MMP-1 mRNA and TIMP-1 mRNA in different colon biopsy samples. Positive rates were analyzed by χ^2 test and Spearman correlation analysis was used to study the relationship between MMP-1 mRNA, TIMP-1 mRNA, MMP-1 mRNA/TIMP-1 mRNA ratio and the severity of clinical symptoms. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using SPSS 10.0 for Windows.

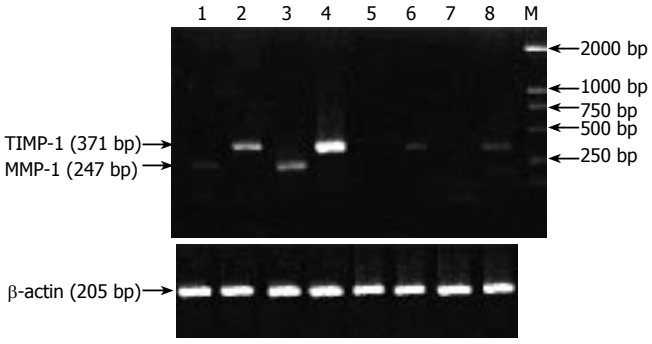

Figure 1 Expression of MMP-1 and TIMP-1 mRNA in UC. Lanes 1, 2: Inflamed area; lanes 3, 4: Ulcerated area; lanes 5, 6: Non-inflamed area; lanes 7, 8: Normal controls; M: DNA marker DL 2000.

RESULTS

Expression of MMP-1 mRNA in UC

Expression of MMP-1 mRNA in the ulcerated and inflamed areas of the colon was significantly higher than that in the non-inflamed areas of the colon of UC patients and normal controls ($P < 0.001$). It was 80-fold higher in the ulcerated area and 30-fold higher in the inflamed area when compared with the normal controls, but there was no statistically significant difference in MMP-1 mRNA expression between UC patients and normal controls ($P > 0.05$, Figure 1, Table 1).

Expression of TIMP-1 mRNA in UC

Expression of TIMP-1 mRNA in the ulcerated and inflamed area of colon was significantly higher than that in the non-inflamed area of colon of UC patients and normal controls ($P < 0.001$). It was 2.2-fold higher in the ulcerated area and 1.6-fold higher in the inflamed area when compared with the normal controls, but there was no statistically significant difference in TIMP-1 mRNA expression between UC patients and normal controls (Figure 1, Table 2).

Correlations between MMP-1mRNA, TIMP-1mRNA, MMP-1 mRNA/TIMP-1 mRNA ratio and the severity of clinical symptoms

Expression of MMP-1 mRNA was significantly related to the expression of TIMP-1 mRNA in the tissues of UC patients, the relating coefficient was 0.801 ($P < 0.001$). Based on clinical manifestations, only 2 patients were classified into severe type, so correlation analysis was performed between moderate group (18 patients) and mild group

Table 1 Expression of MMP-1 mRNA in samples form different areas of colon in UC (mean ± SD)

Samples	MMP-1 mRNA	*P* value
Ulcerated area	0.4136 ± 0.2495	< 0.001[b,d]
Inflamed area	0.1491 ± 0.0891	< 0.001[b,d] < 0.05[a]
Non-inflamed area	0.0102 ± 0.0144	> 0.05
Normal controls	0.0051 ± 0.0086	

[b]$P < 0.001$ *vs* normal controls, [d]$P < 0.001$ *vs* non-inflamed area, [a]$P < 0.05$ *vs* ulcerated area.

Table 2 Expression of TIMP-1 mRNA in samples from different areas of colon in UC (mean ± SD)

Samples	TIMP-1	*P* value
Ulcerated area	0.8512 ± 0.4169	< 0.001[b,d]
Inflamed area	0.7493 ± 0.3505	< 0.05[a,c,e]
Non-inflamed area	0.4434 ± 0.3360	> 0.05
Normal control	0.3903 ± 0.2971	

[b]$P < 0.001$ *vs* normal controls, [d]$P < 0.001$ *vs* non-inflamed area, [a]$P < 0.05$ *vs* normal controls, [c]$P < 0.05$ *vs* non-inflamed area, [e]$P < 0.05$ *vs* ulcerated area.

Table 3 Positive expression rates of MMP-1 in samples from different areas of colon in UC

Samples	Positive cases	Negative cases	Total cases	Positive rate (%)	*P* Value
Ulcerated area	25	5	30	87	< 0.05[a,c]
Inflamed area	25	5	30	87	< 0.05[a,c]
Non-inflamed area	12	18	30	40	> 0.05
Normal controls	7	13	20	35	

[a]$P < 0.05$ *vs* normal controls, [c]$P < 0.05$ *vs* non-inflamed area.

Table 4 Intensity of MMP-1 expression in samples from different areas of colon in UC

Sampes	-	+	+ +	+ + +	*P* value
Ulcerated area	5	2	4	19	< 0.001[a,c]
Inflamed area	5	5	16	4	< 0.05[a,c,e]
Non-inflamed area	18	10	2	0	> 0.05
Normal controls	13	6	1	0	

[a]$P < 0.05$ *vs* normal controls, [c]$P < 0.05$ *vs* non-inflamed area, [e]$P < 0.05$ *vs* ulcerated area.

Table 5 Positive expression rates of TIMP-1 in samples of different areas of colon in UC

Samples	Positive cases	Negative cases	Total cases	Positive rate (%)	*P* value
Ulcerated area	26	4	30	89	> 0.05
Inflamed area	26	4	30	89	> 0.05
Non-inflamed area	24	6	30	80	> 0.05
Normal controls	15	5	20	75	

Table 6 Intensity of TIMP-1 expression in samples from different areas of colon in UC

Samples	-	+	+ +	+ + +	*P* value
Ulcerated area	4	7	9	10	< 0.05[a,c]
Inflamed area	4	8	13	5	< 0.05[a,c,e]
Non-inflamed area	6	19	5	0	> 0.05
Normal controls	5	11	4	0	

[a]$P < 0.05$ *vs* normal controls, [c]$P < 0.05$ *vs* non-inflamed area, [e]$P < 0.05$ *vs* ulcerated area.

(10 patients). The results showed that MMP-1 mRNA, TIMP-1 mRNA and MMP-1 mRNA/TIMP-1 mRNA ratio were all significantly correlated with the severity of the clinical symptoms, and the relating coefficient was 0.411, 0.328 and 0.552, respectively.

Results of immunohistochemistry

Positive expression rates of MMP-1 in the ulcerated and inflamed areas of the colon were significantly higher than those in the non-inflamed areas of the colon of UC patients and normal controls ($P < 0.05$), but there was no statistically significant difference in non-inflamed areas between UC patients and normal controls (Table 3).

The intensity of MMP-1 expression in the ulcerated and inflamed area of colon was significantly higher than that in the non-inflamed area of colon of UC patients and normal controls ($P < 0.001$), but there was no statistically significant difference in the non-inflamed area between UC patients and normal controls (Table 4).

Positive expression rates of TIMP-1 in the ulcerated and inflamed area of colon were not statistically different when compared with those in the non-inflamed area of colon of UC patients and normal controls (Table 5).

The intensity of TIMP-1 expression in the ulcerated and inflamed area of colon was significantly higher than that in the non-inflamed area of colon of UC patients and normal controls ($P < 0.001$). The results also showed that the intensity of TIMP-1 expression in the ulcerated area was significantly greater than that in the inflamed area ($P < 0.05$), but there was no statistically significant difference in the non-inflamed area between UC patients and normal controls (Table 6).

DISCUSSION

UC is a chronic and non-specific inflammatory disease of the colon and affects mainly the colonic mucosa and submucosa. Pathologically, it is characterized by ulceration in the mucosa and submucosa and degradation of ECM is involved in this process. In this study, we separately utilized RT-PCR and immunohistochemistry to detect the expression of MMP-1 and TIMP-1 at both mRNA and protein levels. The expression of MMP-1 mRNA and TIMP-1 mRNA in ulcerated and inflamed areas of colon was significantly higher than that in the non-inflamed area of colon of UC patients and normal controls, the same results were also obtained at the protein level, suggesting that expression of MMP-1 and TIMP-1 is correlated to UC severity at both transcription and protein levels[4]. MMP-1, also termed interstitial collagenase, is able to degrade the

spiral structure of collagen types Ⅰ, Ⅱ, Ⅲ and Ⅹ, making them more sensitive to the hydrolysis of gelatinase, and thus, play an important role in the degradation of ECM[5]. RT-PCR showed that the expression of MMP-1 mRNA was greatly increased in the ulcerated and inflamed areas of the colon of UC patients with the ulcerated area being more profound, suggesting that MMP-1 is related to the mucosal damage[6]. Arihiro *et al*[7] showed that MMP-1 has something to do with the initial steps of ulceration in UC. von Lampe *et al*[3] also found that the expression of MMP-1 is increased by 230-fold in the colonic mucosa of patients with UC compared with normal controls. Meanwhile, our immunohistochemical results showed that MMP-1 protein was significantly increased in the ulcerated and inflamed areas of UC patients. Furthermore, MMP-1 protein was mainly expressed in the interstitial cells. This is similar to the result of von Lampe *et al*[3] who identified that these interstitial cells are macrophages. Mckaig *et al*[8] identified that myofibroblasts are also MMP-1 producing interstitial cells in the colon of patients with UC.

TIMPs are natural inhibitors of MMPs and exert important regulating functions on MMPs. TIMP-1 mainly inhibits the activity of MMP-1, -3 and -9. Our results showed that the expression of TIMP-1 mRNA in the ulcerated and inflamed areas was significantly higher than that in the normal controls, suggesting that the increased expression of MMP-1 up-regulates TIMP-1, leading to an imbalanced state of MMP-1 mRNA/TIMP-1 mRNA ratio, or in other terms, the increased TIMP-1 mRNA was not able to counteract the increased MMP-1 mRNA, resulting in over degradation of ECM in UC. The similar result was also obtained when TIMP-1 protein was measured. Both MMP-1 and TIMP-1 were expressed in the interstitial cells, indicating that MMP-1 and TIMP-1 come from the same cells[2]. Immuno-electron microscopy reveals that MMP-1 and TIMP-1 are localized in the rough endoplasmic reticulum of the activated myofibroblasts and smooth muscle cells of the blood vessels[7], suggesting that MMP-1 and TIMP-1 have something to do with the formation of new blood vessels.

Based on clinical manifestations, only 2 UC patients were classified as severe type in our study. We analyzed the relationship between MMP-1 mRNA and TIMP-1 mRNA expression and the severity of the disease. Our results showed that expression of MMP-1 mRNA and TIMP-1 mRNA in patients with moderate UC was significantly higher than that in patients with mild UC. MMP-1 mRNA, TIMP-1 mRNA and MMP-1 mRNA/TIMP-1 mRNA ratio all were closely related to the severity of the clinical manifestations. Therefore, it is concluded preliminarily that MMP-1 mRNA, TIMP-1 mRNA and MMP-1 mRNA/TIMP-1 mRNA ratio can be used as the parameters in judging the clinical severity of patients with UC. von Lampe *et al*[3] found that MMP-1 mRNA expression is correlated with the pathological staging of inflammation. Wiercinska-Drapalo *et al*[9] found that serum TIMP-1 level is positively correlated with the extent of endoscopic mucosal injury, clinical severity and concentration of C reactive protein in UC patients.

In conclusion, excessive expression of MMP-1 in the diseased colon mucosa of UC patients causes excessive hydrolysis of the ECM and ulceration. MMP-1 mRNA, TIMP-1 mRNA and MMP-1 mRNA/TIMP-1 mRNA ratio can be used as biomarkers to judge the severity of clinical symptoms in patients with UC. Exogenous TIMP-1 or MMP-1 inhibitor therapy is a novel treatment for patients with UC.

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RAPID COMMUNICATION

Genotype-dependent activation or repression of HBV enhancer Ⅱ by transcription factor COUP-TF1

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Abstract

AIM: To study the expression of HBV enhancer Ⅱ by transcription factor COUP-TF1.

METHODS: In order to study the regulation of HBV variants in the vicinity of the NRRE we cloned luciferase constructs containing the HBV enhancer Ⅱ from variants and from HBV genotypes A and D and cotransfected them together with expression vectors for COUP-TF1 into HepG2 cells.

RESULTS: Our findings show that enhancer Ⅱ of HBV genotype A is also repressed by COUP-TF1. In contrast, two different enhancer Ⅱ constructs of HBV genotype D were activated by COUP-TF1. The activation was independent of the NRRE because a natural variant with a deletion of nt 1763-1770 was still activated by COUP-TF1.

CONCLUSION: Regulation of transcription of the HBV genome seems to differ among HBV genomes derived from different genotypes. These differences in transcriptional control among HBV genotypes may be the molecular basis for differences in the clinical course among HBV genotypes.

Key words: Hepatitis B virus; Hepatitis B virus x protein; COUP-TF1; Genotype; Enhancer Ⅱ

Fischer SF, Schmidt K, Fiedler N, Glebe D, Schüttler C, Sun J, Gerlich WH, Repp R, Schaefer S. Genotype-dependent activation or repression of HBV enhancer Ⅱ by transcription factor COUP-TF1. *World J Gastroenterol* 2006; 12(37): 6054-6058

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INTRODUCTION

Hepatitis B virus (HBV) is a major health burden for the world[1]. In an otherwise immune competent host, HBV concentrations of 10^{12} to 10^{13} genome equivalents (GE)/l are frequently found. Thus, in a chronic carrier up to 10^{13} virions are produced per day[2]. Due to the high replication capacity[2,3] and the high error rate of the viral polymerase, HBV genomes with all possible single mutations and double mutations of every nucleotide of the HBV genome are produced every day[3]. Variants of HBV with point mutations, deletions or insertions have been described all over the genome of HBV[4-7]. In addition to viral variants the variability of the virus is increased by the divergence of HBV into 8 genotypes A-H that differ by at least 8% when comparing whole genomes[8-10]. The transcription of the hepadnaviral pregenomic (pG) and precore (pC) RNA is regulated by transcription factors binding to a region containing the enhancer Ⅱ (EII) and the core promoter (Figure 1A)[11]. The expression of both pG and pC RNA is tightly coupled[11], however, natural variants[12] and point mutations[13] located in this transcriptional element can uncouple the transcription of these RNAs. The variants and artificial point mutations that uncouple the transcription of the pG and pC RNA affect a site in this transcriptional element called the nuclear receptor responsive element (NRRE)[13,14]. COUP-TF, a member of the nuclear receptor family[15], binds to the NRRE and has been found to repress transcription from HBV enhancer Ⅱ/pC/pG promoter[13].

We have previously characterised an outbreak of HBV in children in a department of oncology[16,17]. In 1992 we observed a deletion of nt 1763 to 1770 affecting the c-terminus of HBx, enhancer Ⅱ/pC- and pG promoter[18] in serum from a single patient. Later on when we examined serum from 20 patients by amplification of the whole genome from different time points after infection, three

additional patients were found to have the deletion of 8 bp in enhancer Ⅱ[19]. This deletion was found mainly in sera from time points late after infection. The deletion of nt 1763-1770 was found to increase the ratio of pG to pC RNA, the replication of HBV *in vitro*, and to generate a new binding site for the transcription factor HNF-1[12]. However, the deletion of nt 1763-1770 also deletes large parts of the binding site for COUP-TF, which was mapped to 1755-1768[20-22]. We thus analysed whether enhanced replication from variant enhancer Ⅱ/pC/pG promoter may also be caused by the deletion of the binding site for the repressor COUP-TF1. In contrast to previous studies we used HBV genome isolates with different genotype backgrounds and observed significant differences.

MATERIALS AND METHODS

Transfection and luciferase-assay

All experiments were performed with the differentiated human hepatoblastoma line HepG2[23] cultivated in RPMI1640/10% FCS. The different expression constructs were used in luciferase assays. Briefly, 1-1.5 × 10^6 seeded cells were transfected with 1.5 μg luciferase construct and 3 μg of the COUP-TF1 or HNF-1 expression constructs using Lipofectamine (Life Technologies) according to the manufacturers description. All plasmids used were endotoxin free purified (EndoFree, Quiagen). After 48 h the transfected cells were harvested and lysed in Tris-buffer (250 mmol/L, pH 7.8) by 3 cycles of freezing and thawing. Protein concentration of lysates was determined by BCA-assay (Pierce). 15 μg of lysate protein adjusted to 50 μL with Tris-buffer were used in the luciferase activity assay[24]. The results are shown as factors relative to basal luciferase expression and represent the mean values of three independent transfections.

Plasmids

All constructs for reporter assays were cloned into pLuci3 (Promega). The derived constructs were controlled by sequencing. pLEII-A-991 and pLEII-D-Ari: contain the enhancer II/core promoter sequence of genotype A (GenBank: X51970) or D (GenBank: Y07587) from nt 1400 to 1903 and have been described in[25]. For pLEII-D-2.2.15 nt 1400 to 1903 were amplified from supernatants of the HBV expressing cell line HepG2.2.15[26] and cloned accordingly. The sequence of this fragment was identical to GenBank U95551. HBV enhancer Ⅱ, genotype D, with a corresponding truncated construct pL-EII (1730-1822). Derived from this sequence, constructs with an 8bp deletion of nt 1763-1770 or the frequently observed double mutation of 1762 (A/T), 1764 (G/T) have been cloned respectively (pL-EII(1730-1822)d8bp and pL-EII(1730-1822)A/T). For cotransfection expression vectors for HNF1[27] or COUP-TF1[28] were employed.

Electrophoretic mobility shift assay (EMSA)

EMSAs were performed as described by[24] using nuclear extracts from HepG2 cells. For EMSAs the following oligonucleotides with the corresponding binding sites were used: wt (HBV-1755-1805-bs-s): 5'-GAT CCT TAG GTT AAA GGT CTT TGT ATT AGG AGG CTG TAG GCA TAA ATT GGT CTG CGA-3'; wt (HBV-1755-1805-bs-as) 5'-GAT CTC GCA GAC CAA TTT ATG CCT ACA GCC TCC TAA TAC AAA GAC CTT TAA CCT AAG-3'; 8bp-Deletion (bs-HBVd8-1755-1805-s) 5' -GAT CTT AGG TTA AAT ATT AGG AGG CTG TAG GCA TAA ATT GGT CTG CGC A-3'; 8bp-Deletion (bs-HBVd8-1755-1805-as 5'-GAT CTG CGC AGA CCA ATT TAT GCC TAC AGC CTC CTA ATA TTT AAC CTA A-3'; 8bp-Deletion (HBVd8-dTBP-1755-1805-s) 5' -GAT CTT AGG TTA AAT ATT AGG AGG CTG TAG GCA TAA ATT GGT CTG CGC A-3'.

RESULTS

To analyse the effect of COUP-TF1 on a frequently found natural variant of the HBV EII/pC/pG promoter we cotransfected expression constructs for HNF1 and COUP-TF1 together with a luciferase reporter construct containing nt 1730-1817 (pL-pC-D-Ari) or nt 1730-1902 (pL-preG-D-Ari) (Figure 1A-C) into HepG2 cells as described[25].

HNF1 showed a very weak transactivation of both promoter constructs irrespective if wt or the deletion construct were employed. COUP-TF1 showed a strong transactivation of all constructs. It appeared as if the transactivation was higher when constructs that contained the deletion of nt 1763-1770 were used. Two aspects of the latter results were surprising: COUP did not repress transcription from the wt-pG promoter as expected from previously published data[13] and the deletion of a large part of the binding site for COUP in the Δ8bp-pG and -pC construct had no effect on the activation by COUP.

Our results for the pG and pC construct (nt 1730-1900) were seemingly in conflict with the data of[13] who reported a repression of nt 1443 to 1990 of HBV by COUP. Thus, we cloned nt 1400 to 1902 into luciferase constructs. Figure 1C shows that COUP-TF1 also activated expression from the complete enhancer Ⅱ of the isolates described in[18,19,29]. No upstream element seemed to influence the activation of enhancer Ⅱ by COUP-TF1 because all deletion constructs of pLEII-D-Ari and pLEII-D-Ari-Δ8bp were activated by COUP-TF1 (Figure 1).

Because pLEII-D-Ari was cloned from serum of patients infected during massive immune suppression and HBV genomes from these patients are known to contain many variants[19], we analysed the effect of COUP-TF1 on enhancer Ⅱ from an assumed wt-HBV genotype D genome. Figure 2 shows that this construct was also activated by COUP-TF1 even stronger than pLEII-D-Ari. In comparison we analysed a similar construct cloned from genotype A[25]. Quite in contrast to the activation of pLEII-D-Ari, the genotype A construct pLEII-A991 was repressed by COUP-TF as reported for a construct containing enhancer Ⅱ of HBV genotype C[13].

COUP-TF1 is known to exert its repressive effect on HBV enhancer Ⅱ of HBV genotype C through the NRRE around nucleotide 1755-1768[13,14]. However, activation of HBV EII by COUP-TF1 also was observed when we tested constructs with a deletion of nt 1763-1770.

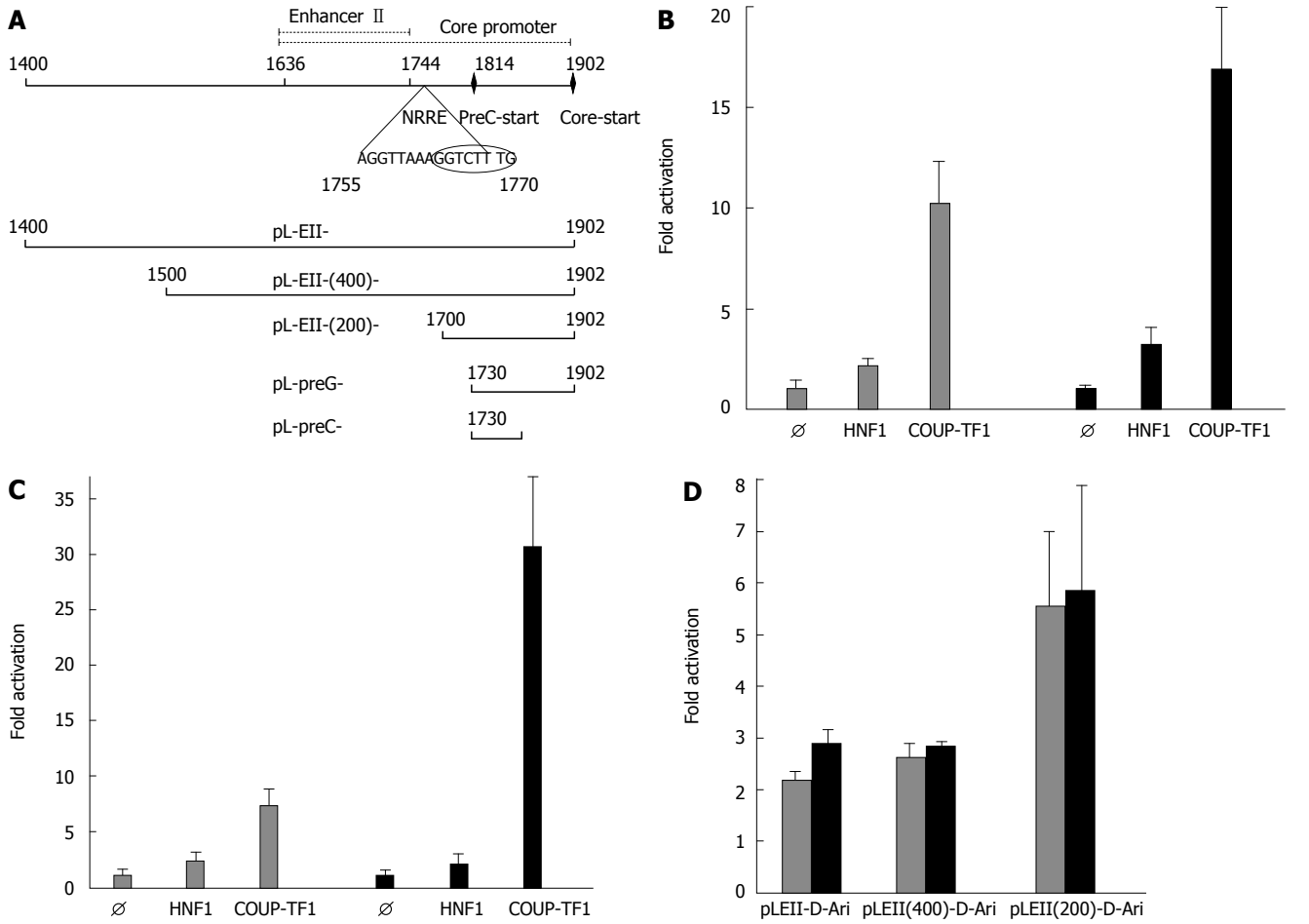

Figure 1 Structure (**A**) and activation of the precore (**B**), the pregenomic (**C**) and enhancer II (**D**) of HBV genotype D by COUP-TF1. **A**: Schematic structure of HBV enhancer II and the core promoter. The sequence of the nuclear receptor response element (NRRE) and the natural deletion of nt 1763-1770 (encircled) is indicated. Below the transcriptional elements the HBV fragments cloned into the luciferase reporter vector pGL3 are shown. For cloning the indicated nucleotides from two plasmids containing wt (GenBank: Y07587) and HBV with a deletion of nt 1763-1770[18] were amplified and cloned. HepG2 cells were transfected with luciferase reporter constructs containing pregenomic promoter (pL-preG-D-Ari, wt in grey); **B**: Precore promoter (pL-preC-D-Ari, wt in grey) **C**: The complete and truncated wt enhancer II **D**: (pLEII-D-Ari, in grey) corresponding variant constructs with a deletion of nt 1763-1770 (Δ8bp) in black. For cotransfection the empty expression vector (Ø) or expression vectors for HNF1[27] or COUP-TF1[28] were employed.

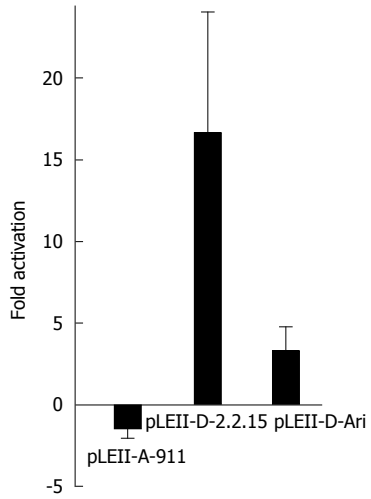

Figure 2 Influence of the nuclear receptor COUP-TF1 on enhancerII/core promoter constructs of different HBV genotypes. HepG2 cells were transfected with luciferase constructs for the complete enhancer II (1400-1902) of genotype A (pLEII-A-991) or two constructs (pLEII-D-Ari and pLEII-D-2.2.15) derived from genotype D and the expression vector for COUP-TF1.

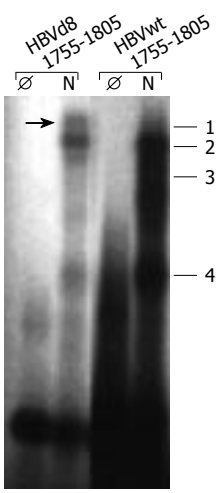

Figure 3 Deletion of nt 1763-1770 leads to altered band shifts in electrophoretic mobility shift assay with oligonucleotides nt 1755-1805. EMSA was performed as described by[25] with oligonucleotide nt 1755-1805 wt (HBVwt 1755-1805) and with a deletion of nt 1763-1770 (HBVd8 1755-1805) incubated with (N) or without (Ø) nuclear extract prepared from HepG2 cells with a method[25].

Thus, activation by COUP-TF1 may be exerted through other binding sites. To study the binding of the NRRE by cellular proteins we performed EMSAs with nuclear extracts prepared from HepG2 cells as described in[24]. Figure 3 shows that the wild type oligonucleotide was bound by several proteins purified from nuclear extracts of the human hepatocyte line HepG2. Four bands could be discerned in the EMSA with nuclear extract. Band 2 and 4 were common to wt and the 8 bp deletion. Band 1 was unique to the variant, band 3 to the wt.

DISCUSSION

Our results show that the ubiquitous transcription factor COUP-TF1 was a strong activator of wt and variant pG, pC promoter and enhancer Ⅱ constructs (Figure 1 and 2) derived from sera of a single source outbreak[18,19,29]. This result was surprising because Yu *et al*[13] reported that the wt-enhancer Ⅱ (nt 1443 to 1990) was repressed by COUP-TF1. The involvement of distal elements in enhancer Ⅱ that may be needed for activation by COUP-TF1 is unlikely because we did not observe a change in activation by deletion of upstream sequences (Figure 1D).

The NRRE seems to be dispensable for activation by COUP-TF1 in our genotype D constructs because all constructs containing a deletion of nt 1763-1770 were activated by COUP-TF1 as well as the wt-constructs (Figure 1). In addition, no binding of COUP-TF1 was observed by EMSA when we used an oligonucleotide containing a frequently found deletion of nt 1763-1770 (data not shown). These data support the findings of Yu and Mertz who reported that two frequently occurring natural point mutations of nt 1764 and 1766 also abolish binding by COUP-TF1[14].

Our data are compatible with the report from the group of Mertz if HBV genotype differences are taken into consideration. Yu *et al*[13] found that the NRRE from nt 1755-1768 was essential for the repression of transcription from HBV enhancer Ⅱ, pC and pG promoter of genotype C[13,14]. We have analysed activation of HBV enhancer Ⅱ, pC and pG promoter of genotype D by COUP-TF1. For activation by COUP-TF1 nt 1763 to 1770 of the NRRE seem to be dispensable. Thus, our data imply that activation of HBV enhancer Ⅱ, pC and pG promoter is regulated by other elements than the NRRE. We can not rule out the possibility that COUP-TF1 exerts its activation of enhancer Ⅱ of HBV genotype D by indirect mechanisms not involving direct binding to the promoter as in the activation of the vHNF1 promoter[30].

Further analyses with constructs containing HBV DNA from other sources showed that very similar constructs of HBV enhancer Ⅱ reacted differently to COUP-TF1: constructs derived from genotype A (Figure 3) or C [13] were repressed by COUP-TF1 whereas constructs of genotype D derived from two different sera were activated. A sequence analysis (data not shown) of these four constructs revealed no differences in the NRRE at bp 1755 – 1768, which has been shown to be essential for repression by COUP-TF1[13,14]. However, the sequences upstream of the NRRE showed sequence variability as expected for HBV isolates of different genotypes. However, we were not able to detect a sequence motif that may be indicative for activation or repression by COUP-TF1.

HBV genotypes influence the course and outcome of preventive and therapeutic measures[8-10,31-33]. Very limited data are available on the effect of the sequence variability on *in vitro* properties of HBV, which may explain the different outcome of HBV infections depending on the genotype of HBV. In a relatively large analysis using HBV constructs of HBV genotypes A, C, D and E, Sommer *et al* observed differences in splicing of the HBV pregenome[34]. Other groups observed higher repression of apoptosis by HBx derived from genotypes D compared to HBx of genotype C origin[35]. Using another system, HBx of genotype D also showed a higher activity than HBx of genotype A in the induction of apoptosis[36].

Thus, the variability induced by HBV genotypes may result in different molecular biology of HBV. However, there is more research to be done because our current results do not answer the question whether the differences described by several groups can be attributed to properties conserved in a given genotype or only represent certain variants that may occur in all 8 HBV genotypes.

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CASE REPORT

Necrosis of a large hepatic tumor after hemorrhage and subsequent selective arterial embolization

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Abstract

This case report describes a young female patient presenting with acute intra-abdominal hemorrhage originating from a large tumor in the liver, most likely a hepatocellular adenoma. The bleeding was stopped by selective embolization of right hepatic artery branches. Subsequently, partial hepatectomy was performed after 6 mo. Macro- and microscopic examination showed complete necrosis and absence of tumorous tissue. The patient was discharged without complications, and subsequent follow-up until 22 mo after resection did not reveal any new lesions in the liver. This case emphasizes the significance of selective arterial embolization in the management of bleeding liver tumors and questions the need for (partial) hepatectomy after this procedure in selective cases.

Key words: Liver hemorrhage; Selective arterial embolization; Hepatocellular adenoma

Huurman VAL, Stoot JHMB, van der Linden E, Terpstra OT, Schaapherder AFM. Necrosis of a large hepatic tumor after hemorrhage and subsequent selective arterial embolization. *World J Gastroenterol* 2006; 12(37): 6059-6061

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INTRODUCTION

Hepatocellular adenomas are uncommon benign liver tumours that are present mainly in women of reproductive age[1]. Their existence is associated with the use of oral contraceptives[2]. The first clinical presentation of the tumour can consist of abdominal pain, hepatomegaly, or hepatic (subcapsular) hemorrhage and shock[1]. For the latter presentation, emergency surgery by partial hepatectomy is the current treatment of choice. However, this treatment is associated with high morbidity and mortality rates[3,4]. Therefore, several groups suggest a role for initial conservative treatment[1,5].

Selective arterial embolization is a novel method for the management of intra-abdominal hemorrhage without the need for major surgery, and our hospital has gained considerable experience with this procedure. However, the use of this elegant method when coping with liver hemorrhage has only been described incidentally[1,5,6].

After any initial emergency treatment, secondary partial hepatectomy is generally recommended in patients with lesions $\geqslant$ 5 cm, because of increased risk of rupture as well as malignant degeneration[1,5]. The case we present, however, suggests a role of selective arterial embolization not only in the primary emergency situation, but also as a therapeutic tool to achieve necrosis of an arterially vascularized liver tumor.

CASE REPORT

A 35-year old woman was referred to our hospital with a two-day history of right abdominal pain without any previous trauma. The patient had no medical history, her medication consisted of oral contraceptive treatment only (30 μg ethinylestradiol, 150 μg levonorgestrel, taken during eleven years). Despite initial fluid supplementation, her heart rate was elevated (110/min) with normal blood pressure. Further physical examination showed tenderness of the right upper abdomen. Hemoglobin levels were low (78 g/L). INR was 1.0, APTT 21.5 s, PTT 12.5 s and thrombocytes 213 $\times$ 10^9/L. Serum liver enzyme levels were elevated (ASAT 550 U/L, ALAT 607 U/L, Alk.Fos 75 U/L, γGT 109 U/L, LDH 758 U/L). She was transfused with 2 units of red blood cells. Computed tomography (CT) scan of the abdomen revealed a large subcapsular hematoma surrounding a tumorous mass in segment Ⅳ-Ⅶ of the liver, as well as intraperitoneal fluid. Arteriography confirmed hemorrhage originating from the right hepatic artery, which was also responsible for the vascularization of the tumor. Subsequently, selective arterial embolization of the majority of right hepatic artery branches was performed in order to stop the bleeding using both polyvinyl alcohol (PVA) particles and a mixture of enbucrilate (histoacryl) and

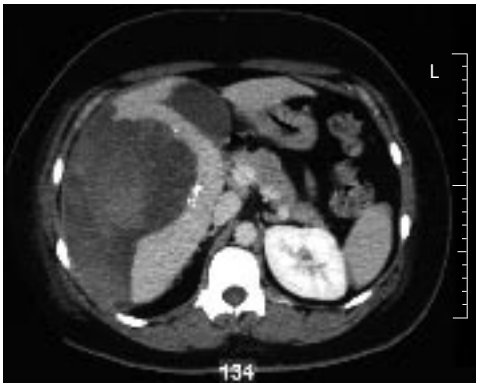

Figure 1 Contrast-enhanced computed tomography (CT) scan of the abdomen 6 d after selective arterial embolization, showing liver hemorrhage with a circumscribed mass as its conceivable origin.

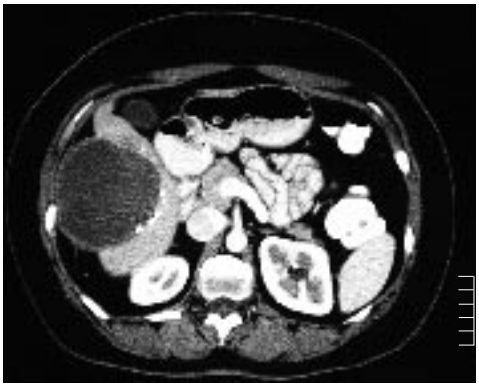

Figure 2 Arterial phase CT scan of the abdomen before partial hepatectomy showing remains of the hematoma and the known circumscribed mass, which is not vascularized arterially. Note the arterial deposition of histoacryl and lipiodol along the tumor as a result of selective embolization.

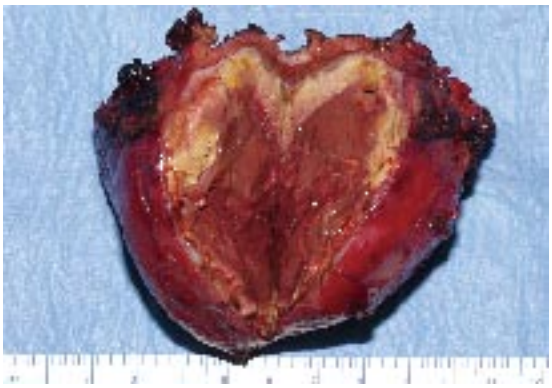

Figure 3 Macroscopic view of the resected tumor (∅ 7 cm) consisting entirely of necrotic material.

iodized poppy-seed oil (lipiodol). Contrasr-enhanced CT scan 6 d after embolization clearly showed a circumscribed tumorous mass (∅ 7 cm) in segment V of the liver, most likely a hepatocellular adenoma (Figure 1). Further hospital stay was unremarkable and the patient was discharged 11 d after admission. Following our department's protocol on liver adenomas with a diameter ⩾ 5 cm, elective resection of segment V was performed six months later. Preoperative contrast-enhanced CT scan showed the remains of both the hematoma and the known circumscribed mass (Figure 2). Arterial perfusion of segment V of the liver was absent whereas portal perfusion was intact. The resected tumor consisted completely of necrotic material (Figure 3). No clear tumor classification could be made by histological examination due to absence of tumorous material, but the macroscopic signs of necrosis were confirmed. Tumor markers (CEA, CA 19.9 and α-foetoprotein) were in the normal range. The patient was discharged without complications, and subsequent follow-up until 22 mo after resection did not reveal any new lesions in the liver. She was advised to refrain from oral contraceptive medication.

DISCUSSION

The increased chance of rupture of large hepatocellular adenomas makes the presence of such a tumour a considerable potential hazard. Management of this condition still needs improvement. The presence of a ruptured hepatocellular adenoma cannot be proven by CT scan alone, but is strongly suggested by our patient's clinical presentation. Although hepatocellular carcinoma and focal nodular hyperplasia lesions have also been described as causes of liver hemorrhage[7], their presence is unlikely in this patient. The presence of liver adenomas in young women is associated with the use of oral contraceptives. In this case no specific histologic tumor classification could be made, since cessation of arterial blood flow by embolization caused necrosis of the tumor. This corresponds with the fact that hepatocellular adenomas are only vascularized arterially. It has to be noted that regression of liver adenomas is possible not only after ischemia, but also after hormone withdrawal[8], hemodialysis[9] and dietary therapy for glycogen storage disease[10].

Embolization is safe and successful in stopping the hemorrhage. The necrosis of the tumor may point to a future role of selective arterial embolization in the management of either bleeding or non-bleeding hepatocellular adenomas. Furthermore, this case argues in favour of conservative follow-up after embolization, regardless of the initial size of the adenoma. This would further limit the indications for surgery in a selected group of patients, resulting in reduction of patient morbidity and mortality.

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CASE REPORT

Biliary cystadenoma with mesenchymal stroma: Report of a case and review of the literature

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Abstract

Biliary cystadenomas are rare, cystic neoplasms of the biliary ductal system that usually occur in middle-aged women. They cannot be safely differentiated from cystadenocarcinomas before operation and should always be considered for resection. Cystadenomas have a strong tendency to recur, particularly following incomplete excision, and a potential of malignant transformation. Therefore, complete resection is the therapy of choice and thorough histopathologic evaluation is imperative. A case of benign biliary cystadenoma with mesenchymal stroma is presented along with a review of the relative literature addressing the clinical presentation, histology, histogenesis, differential diagnosis, imaging features, treatment and prognosis of this interesting and rare entity.

Key words: Biliary cystadenoma; Biliary cystadenocarcinoma; Mesenchymal stroma

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INTRODUCTION

Biliary cystadenomas are rare, benign but potentially malignant, multilocular, cystic neoplasms of the biliary ductal system[1,2]. They usually arise in the liver (80%-85%)[2,3], less frequently in the extrahepatic bile ducts[2,3] and rarely in the gallbladder[4], accounting for less than 5% of cystic neoplasms of the liver[5,6]. Less than 100 reports of intrahepatic biliary cystadenomas are identified in the medical literature[5,7-10].

They more frequently occur in middle-aged women[7,10,11]. The patients may be asymptomatic with their tumors discovered incidentally during radiographic evaluation or surgical exploration for other clinical indications[6]. However, they often have vague abdominal complaints related to extrinsic compression of the stomach, duodenum, or biliary tree[5,7,12]. The premalignant nature or potential for malignant transformation or degeneration and the tendency to recur, particularly when treated with techniques other than complete excision, are of great concern in these tumors[7,10,11]. Furthermore, since clinical presentation as well as laboratory and imaging data are highly variable and non specific, cystadenoma cannot be differentiated preoperatively from cystadenocarcinoma[1,5,6,10,13].

Such entities in surgical pathology are presented mainly as case reports or retrospective collective studies since their rarity prohibits a prospective evaluation. A case of benign biliary cystadenoma with mesenchymal stroma is presented here along with a literature review.

CASE REPORT

A 39-year old woman complained of vague abdominal pain and increasing abdominal girth. No other signs or symptoms were discovered on clinical examination apart from marked hepatomegaly. All blood tests were normal including liver function tests and levels of serous neoplasmatic markers such as carbohydrate antigen 19-9 (CA 19-9), carcinoembryonic antigen (CEA), carbohydrate antigen 125 (CA 125), and α-fetoprotein (AFP). Anti-echinoccocal IgM and IgG antibodies and viral markers for hepatitis B and C were negative. Ultrasound (US) finding of a cystic lesion of the liver led to a computed tomography (CT) scan that revealed a large (19 cm × 16 cm × 10 cm), well-encapsulated, binodular, cystic formation of the liver occupying most of the right lobe (Figure 1). The lesion appeared to exceed the limits of the liver, reaching the right iliac fossa. A radiological diagnosis of hydatid cyst was offered.

The patient underwent laparotomy under an extended right subcostal incision that revealed a large cystic formation with no macroscopic resemblance to a hydatid cyst. A decision for right hepatic lobectomy was made and segments V-Ⅷ were excised under proximal vascular control. No intraoperative blood transfusion was required. The postoperative course was uneventful and the patient

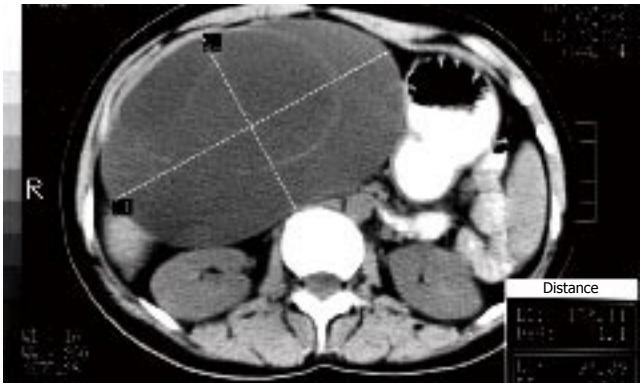

Figure 1 Abdominal CT scan revealing a large binodular cyst occupying most of the right liver lobe.

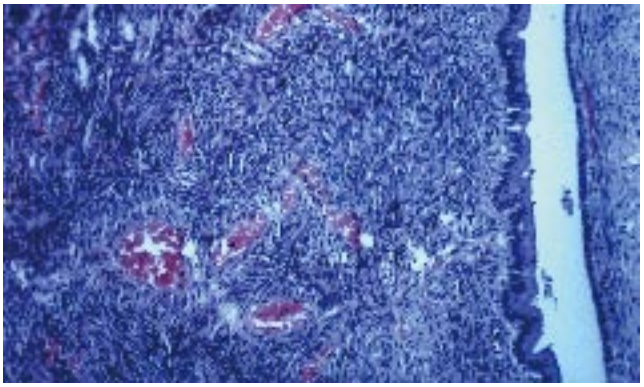

Figure 2 Histologic appearance of the simple columnar and cuboidal epithelium that is typical of biliary cystadenoma. The underlying mesenchymal stroma is densely cellular resembling ovarian.

was discharged six days after operation.

The cytology report of the cystic fluid identified clusters of glandular cells with round nuclei and clear cytoplasm. The most possible diagnosis was biliary cystadenoma. The final histology report referred to an 18 cm × 16 cm gray-white cystic lesion with 0.1-0.3 cm of wall thickness indicating intramural microcystic formation. The lesion was characterized as a hepatobiliary cystadenoma. The epithelium was simple columnar and cuboidal with no atypia and had an underlying densely cellular mesenchymal stroma resembling ovarian (Figure 2). Histological examination showed complete removal of a benign hepatobiliary cystadenoma with mesenchymal stroma.

The patient has remained free of disease for a period of 4 years on repeated CT scans since she was discharged.

DISCUSSION

Cystadenomas are rare, benign but potentially malignant, multilocular, cystic neoplasms of the biliary ductal system[1,2,7,10], accounting for less than 5% of cystic neoplasms of the liver[5,6,10]. The incidence of intrahepatic biliary cystadenomas is between one in 20 000 to one in 100 000 people while the incidence of cystadenocarcinomas approximately one per 10 million patients[14]. Hepatobiliary cystadenomas can occur at any age[15], but they are usually seen in middle-aged women[7,10,11,16]. Approximately 85%-95% of the patients are women[12,15,17]. Even though they are true proliferative epithelial tumors, their progression is characteristically slow and years are required before they enlarge[11]. Their size is variable ranging from 1.5 to 30 cm in diameter[2,5,11,16,18]. They usually arise in liver (80%-85%)[2,3,7,10,19,20], less frequently in extrahepatic bile ducts[2,3,7,10,17,19] and rarely in gallbladder[4]. Approximately 50%-55% of cystadenomas are located in the right lobe with the remaining located in the left lobe or in both lobes (30%-40% in the left lobe and about 15%-20% in both lobes) while few arise from extrahepatic ducts[2,11,12,21]. Accordingly, the common or the right bile duct can be identified as the origin in more than half of cases.

Although reports of cystadenomas have been increasing due to advances in imaging diagnoses and the common use of US and CT, less than 100 reports of intrahepatic biliary cystadenomas are identified in the literature[5,7-10,22] and only 26 extrahepatic cystadenomas have been reported[17,23]. A review of the literature is provided along with a discussion addressing the clinical presentation, histology, histogenesis, differential diagnosis, imaging features, treatment and prognosis for this rare entity.

Clinical presentation

The patients may be asymptomatic with their tumor discovered incidentally during radiographic evaluation or surgical exploration for other clinical indications or even at autopsy[6,24]. However, they often have vague abdominal complaints related to extrinsic compression of adjacent structures such as the stomach, duodenum, or biliary tree. Symptomatic patients present with an insidious onset of symptoms due to the slowly growing nature of the neoplasm[12].

The typical patient is a white female presenting with abdominal discomfort, swelling, gradual increase in abdominal girth and/or pain and a palpable abdominal mass[5,7,11,12]. Right upper quadrant or epigastric pain along with increasing abdominal girth or awareness of an abdominal mass are the main complaints in about 60% of the patients as reviews of the reported cases have shown[21]. In another series, complaints include abdominal pain in 74%, abdominal distension in 26%, and nausea/vomiting in 11% of the patients[25]. The patients may less frequently have gastrointestinal obstruction leading to nausea and vomiting, dyspepsia, anorexia, weight loss or ascites[11,26].

When complicated, symptoms include biliary obstruction[5,7,12], rupture[19], bacterial infection[19], intracystic hemorrhage[19,22] or malignant transformation[7,10]. Biliary obstruction may be either due to the tumor itself[21,27] or either, rarely, due to the secretion of mucin[20,28] and is manifested as jaundice, biliary colic, cholangitis, nausea, fever, chills, itching, or steatorrhea[22]. Such cases represent approximately 35% of the patients with cystadenomas[24]. Patients may experience intermittent jaundice or repetitive episodes of biliary colic or cholangitis for a long period of time before the diagnosis[21,22]. Obstructive jaundice,

although not always present[29], is the most frequent presenting symptom in patients with extrahepatic cystadenomas[17,19]. Other reported symptoms are right upper quadrant or epigastric pain, nausea, vomiting, skin itching and occasional diarrhea[29]. On the contrary, in intrahepatic cystadenomas, biliary obstruction is rarely the chief presenting complaint of the patients[22]. Any patient with clinical or biochemical obstructive jaundice of unknown etiology or recurrence of a liver cyst following surgical treatment should be suspected of having an intrahepatic or extrahepatic cystadenoma[28-30] or a cystaden ocarcinoma[5,10].

Histology

Biliary cystadenomas are usually large, multiloculated, with internal septation and nodularity and surrounded by a dense cellular fibrostroma. Although it may be extremely rarely be unilocular[18,22], multilocularity of the tumor is a key feature that distinguishes cystadenomas from developmental cysts[16]. Biliary cystadenomas are usually globular, with a smooth external surface and a smooth or trabeculated inner surface and contain locules of variable sizes[10,16]. The internal surface of the cyst may also have papillary infoldings or smaller cysts[10]. On light microscopy, cystadenoma consists of three layers: a cyst lining of biliary-type epithelium, a moderately-to-densely cellular stroma, and a dense layer of collagenous connective tissue[31].

The cyst wall consists of a single layer which is typical of biliary-type epithelium containing cuboidal-to-tall columnar and nonciliated mucin secreting epithelial cells with papillary progressions, pale eosinophilic cytoplasm and basally oriented nuclei. The cyst usually contains clear mucinous fluid[10,19] which raises the possibility of a malignant component when hemorrhage occurs[4,11], unless there is a trauma history. Rarely, the fluid within the cyst may be bilious, purulent, proteinaceous, gelatinous, clear or mixed. The septa of the tumor may show calcification.

Foci of epithelial atypia or dysplasia consisting of nuclear enlargement and hyperchromasia, multilayer, loss of polarity, and mitotic activity indicate potential malignant changes[7,16]. Anaplasia or pleomorphism, severe architectural atypia (such as exophytic papillae) and capsular or stromal invasion are features of malignancy[5,10,16]. The premalignant progression is usually based on the histologic presence of intestinal metaplasia which is characterized by the presence of numerous goblet cells[7]. Careful pathologic evaluation is emphasized since malignant degeneration or transformation can be detected only after thorough sectioning. Histological differentiation of biliary cystadenoma from other cystic liver lesions is usually based on its multilocularity, columnar epithelium, papillary infoldings, and ovarian-like stroma[16].

Similar lesions occur in the pancreas and ovary. Two histological variants of biliary cystadenoma are recognized, a serous type and a far more common mucinous type[16]. The rare serous variety resembles serous cystadenoma of the pancreas and is not known to undergo malignant transformation[16]. The mucinous type is predominantly a tumor of middle-aged women and is similar to mucinous cystadenoma of the pancreas[16].

If the underlying subepithelial stroma is densely cellular resembling ovarian stroma as in our case, it is referred as "mesenchymal stroma"[5,8]. The stromal cells are spindle-shaped and usually immunoreactive with vimentin, alpha-smooth muscle actin, and muscle-specific actin and less frequently with desmin, estrogen and progesterone receptors[8,16,27]. Although mesenchyme of these tumors resembles ovarian stroma morphologically, such immunohistochemical features are characteristic of myofibroblasts[8,31]. Mesenchymal stroma is more often observed in the mucinous type of cystadenoma[16]. There are two distinct types of cystadenomas based on the presence or absence of "mesenchymal stroma"[5]. Cystadenoma with mesenchymal stroma occurs exclusively in women[5,7,8,31,32] while cystadenoma without mesenchymal but with hyaline stroma arises in both men and women and tends to occur in older patients[5,32]. Cystadenoma with mesenchymal stroma is regarded as a precancerous lesion[33] but the type without mesenchymal stroma seems to undergo malignant degeneration much more frequently[32]. Patients with cystadenocarcinoma with mesenchymal stroma have a good prognosis whereas the prognosis of patients with cystadenocarcinoma without mesenchymal stroma is poor, especially in men[7]. The malignancy arises from the epithelial component in most cases[33], although sarcomatous transformation of the mesenchymal stroma has been reported[31,34].

Etiology-histogenesis

The origin of biliary cystadenomas is unclear. Theories on their etiology and histogenesis are not solid since both acquired and congenital origins have been proposed. Experimental studies by Cruickshank *et al*[34] may support the theory of an acquired lesion. The development of the tumor as a reactive process to some focal injury has also been mentioned[10]. Mesenchymal stroma, though resembling ovarian microscopically, is more akin to the primitive mesenchyme in embryonic gallbladder and large bile ducts[8,9], suggesting that the tumor may arise from ectopic embryonal tissue destined to form the gallbladder[9] or from ectopic embryonic rests of primitive foregut sequestered within the liver[5,10,33]. Demonstration of endocrine cells in about 50% of hepatobiliary cystadenomas and cystadenocarcinomas could also suggest an origin from intrahepatic peribiliary glands[35]. The presence of hamartomatous structures and development abnormalities supports the theory of congenital origin in at least some cystadenomas[10,36].

A possible hormonal dependance of cystadenomas with mesenchymal stroma has also been proposed since immunohistochemical studies showing the characteristics of mesenchymal stromal cells, have revealed myofibroblastic phenotype and expression of progesterone and estrogen receptors[27,37,38]. In addition, there are reports of this tumor occurring in oral contraceptive users, suggesting that estrogen-containing oral contraceptives may serve as tumor promoters[11,38]. Ectopic ovarian tissue, however, is considered an unlikely origin of these tumors[5,8].

It is uncertain whether biliary cystadenocarcinomas are *de-novo* cancer or are derived from cystadenomas. They are generally thought, however, to arise from preexisting benign cystadenomas since many cystadenocarcinomas contain areas of cystadenoma in the same sample[5,10,33,39].

Differential diagnosis

In the evaluation of cystic hepatic lesions, a high index of suspicion is imperative. Moreover, since these neoplasms have a strong tendency to recur and undergo malignant transformation, differentiating between cystadenomas and other cystic liver lesions is substantially important. Differential diagnosis includes simple liver cysts, parasitic cysts (particularly hydatid cysts), haematomas or post-traumatic cysts, liver abscesses, congenital cysts, polycystic disease, hamartomas, Caroli's disease, and neoplasmatic lesions such as biliary cystadenocarcinoma, undifferentiated embryonal sarcoma, cystic metastasis, metastatic pancreatic or ovarian cystadenocarcinoma, biliary papilloma, cystic primary hepatocellular carcinoma, cystic cholangiocarcinoma, and hepatobiliary mesenchymal tumors (particularly biliary smooth muscle neoplasms) such as biliary leiomyoma, adenomyoma, and primary hepatic leiomyosarcoma[2,27,40]. Preoperative and intraoperative diagnosis of biliary cystadenomas and cystadenocarcinomas can be very difficult and differentiation between these two entities can be safely done only after histopathologic evaluation[1,5,6,10,33,40].

Specific attention should be paid to liver hydatid disease, especially in countries with a high incidence of the disease. Since its imaging features are similar to those of cystadenoma as in our patient, preoperative differentiation may be impossible without serologic tests[2,24,41]. Anti-echinococcus granulosis and anti-amoebic serologic tests, estimation of CA 19-9, CEA and AFP levels, general evaluation of liver and renal function as well as abdominal US, CT and magnetic resonance imaging (MRI) should be performed. Liver function tests may be normal or elevated in cases of intrahepatic or extrahepatic biliary duct compression[11,27,29].

Although they do not rule out cystadenoma when normal[14], serum Ca 19-9 levels are believed to be a valuable marker in the diagnosis and monitoring in the postoperative follow-up since they are reported to return to normal after complete resection[11,42]. Nevertheless, immunoreactivity for CA 19-9 is lost therefore minimizing the possible benefit of serial follow-up when cystadenoma is transformed to cystadenocarcinoma[43].

Measurement of cyst fluid CA 19-9 and CEA levels has been advocated as an adjunctive preoperative procedure for enhancing the accuracy of differentiation of cystadenomas and cystadenocarcinomas from other hepatic lesions[14,44]. High levels of CA 19-9 and CEA can be encountered in cystic fluid or in epithelial lining of biliary cystadenomas, particularly in those with mesenchymal stroma[5,14,30,41,44] and cystadenocarcinomas[45]. These elevated cyst fluid tumor markers clearly indicate the neoplastic features and biliary origin of these cysts[5]. Elevation of CEA may, however, be moderate or not always present[14]. On the contrary, substantially higher levels of CA 19-9 in cystic fluid are observed in the majority of cystadenomas compared with those in simple cysts, echinococcal cysts, and polycystic liver disease[14,30,41,42,44]. Therefore, though not allowing differentiation between cystadenoma and cystadenocarcinoma, measurement of CA 19-9 in cystic fluid obtained by fine needle aspiration as well as in serum may be helpful in the differential diagnosis of hepatic cystic lesions. Moreover, Koffron *et al*[14] have proposed a diagnostic and therapeutic algorithm for hepatic cysts and particularly intrahepatic biliary cystadenomas based on CA 19-9 and CEA levels and cytology along with laparoscopic cyst wall biopsy.

Percutaneous fine needle aspiration also provides fluid for bilirubin concentration analysis, which is suggestive of communication of cystadenoma with the biliary tract when it is elevated[14], and for cytologic evaluation which may be helpful in excluding hepatic abscess, cystic metastases and other cystic lesions. Although there are reports of cystadenocarcinomas arising from cystadenomas diagnosed by percutaneous fine needle aspiration cytology[45,46], it is not very accurate since this procedure relying on adequate sampling may miss the microscopic foci of the carcinoma in cystadenoma[44,47,48]. Thus, correlation of aspiration cytology with clinical and radiological data has been suggested[14,47]. Fine needle aspiration and needle biopsy for diagnosis, however, may also risk dissemination of tumor cells and is not generally recommended, particularly when surgery is planned[30,48]. Pleural and peritoneal dissemination of tumor cells caused by aspiration has been reported[48,49].

Furthermore, examination of epithelial cells of several types of hepatic cysts by mucin histochemistry and immunohistochemistry reveals different features of these cells regarding mucus and antigenic expression among the hepatic cysts[50]. Epithelial cells of non-parasitic simple cysts and adult-type polycystic liver show similar mucin-histochemical and immunohistochemical features, and are characterized by little mucin and weak immunoreactivities to several antibodies. On the contrary, epithelial cells of cystadenoma and cystadenocarcinoma are characterized by much mucin and moderate to strong immunoreactivities to cytokeratins CAM5.2 and AE1 and AE3 as well as to CA 19-9, CEA and epithelial membrane antigen (EMA)[16,50].

Although frozen sections may be helpful in differentiation of cystadenomas and cystadenocarcinomas from other cystic hepatic lesions[18], they are not very useful due to the variability in histology of cystadenomas and their inability to rule out cystadenocarcinomas[48,51,52]. Careful histopathologic evaluation of the resected specimen, therefore, constitutes the only safe diagnostic modality of hepatobiliary cystadenomas and cystadenocarcinomas while malignant degeneration or transformation of a cystadenoma can only be detected after thorough sectioning.

Imaging features

Diagnosis and differential diagnosis of cystadenoma from other cystic hepatic lesions are mainly based on abdominal US, CT scan and MRI. Preoperative US assessment is almost always performed but cannot replace the

diagnostic value of CT. Moreover, the role of US and CT is considered complementary[53]. By US the tumor appears well-demarcated, thick-walled, noncalcified, anechoic or hypoechoic, globular or ovoid, cystic mass and may present thin internal septations, which are highly echogenic, mural nodules and polypoid or papillary infoldings[2,11,15,24,53]. Dilatation of intrahepatic or extrahepatic bile ducts may also be disclosed[27]. In case of intracystic hemorrhage, US may show a hyperechoic mass with no septation[22].

The need to determine the size, morphology and anatomic relation to surrounding structures, particularly major vessels of the lesion prior to intervention is crucial and CT is of great help for the surgeon. Additionally, CT during arteriography is useful to demonstrate the tumor vascularity[45]. Common features on CT scan include low-density, well-defined, lobulated, multilocular, thick-walled, cystic masses with internal septa and occasionally mural nodules[2,12,15,26,53]. Intravenous contrast on CT enhances the cyst wall and septations. CT may sometimes demonstrate dilatation of intrahepatic or extrahepatic bile ducts. Rarely capsular, mural or septal calcification presents on US or CT[2,15,54]. Although such features can be identified with CT and are essential for differentiating cystadenoma from other cystic liver lesions, a possible preoperative diagnosis is rarely suspected due to the rarity of the disease.

Demonstration of communication between the tumor and the biliary tract is of important diagnostic value both in identifying the site of origin of the tumor and in differentiating biliary cystadenoma/cystadenocarcinoma from other hepatic cystic lesions[54]. Such a communication can be demonstrated with percutaneous transhepatic cholangiography or cystography (PTC), endoscopic retrograde cholangiopancreatography (ERCP), magnetic resonance cholangiopancreatography (MRCP), intraoperative cholangiography, cyst fluid examination for bile content, or surgical exploration[12,14,18,45,54,55]. Moreover, since the potential for cystadenomas to extend intraluminally into extrahepatic ducts has been described[41], preoperative extrahepatic biliary imaging is imperative when surgical management is planned. It is noteworthy that no biliary connection has been identified, even at the time of surgery, in the majority of reported cystadenomas[11]. In a review of the literature, Sato *et al*[45] reported that such a biliary fistula was found in only 21 cystadenomas and 16 cystadenocarcinomas.

ERCP or PTC may show an intraluminal filling defect that may be the tumor or represent intraluminal mucin[20]. An infrequent finding is the endoscopic visualization of mucin being extruded through the ampulla of Vater[18]. PTC can provide further information on biochemical and cytologic examinations of the cyst fluid[45]. However, fine needle aspiration cytology is not very accurate[44,47,48] while aspiration may risk possible tumor pleural or peritoneal seeding[48,49]. Therefore, it should be avoided in cases of obvious or suspected cystadenocarcinoma, especially when surgery is planned[48]. ERCP may also be performed in order to reveal or exclude compression or displacement of intrahepatic and extrahepatic bile ducts and is especially important if patients present with jaundice[19]. Furthermore, diagnosis of a cystadenoma of the extrahepatic biliary tree can be obtained by intraoperative cholangiography and/or choledochoscopy, PTC, ERCP, MRCP or spiral CT scan[21,55-57].

MRI is a valuable tool for the diagnosis and differentiation of cystadenoma from other cystic liver lesions while combination of MRI with MRCP is also even more useful[55]. On T1-weighted images, MRI reveals a fluid-containing, multilocular, septated mass with homogenous low signal intensity, the wall and septa of which become enhanced after administration of Gd-DTPA[2,54,55]. On T2-weighted images the fluid collections within the tumor demonstrate variable, homogenous high signal intensity while the wall of the mass is represented by a low-signal-intensity rim[2,54,55]. Variable signal intensities on T1- and T2-weighted images depend on the presence of solid components, hemorrhage, and protein content[2,54,55]. On T1-weighted images, the signal intensity may change from hypointense to hyperintense while septations may be obscured, and only mild enhancement of the cyst wall is noted after Gd-DTPA administration, as protein concentration and viscosity of the cyst fluid increase. In contrast, on T2-weighted images, signal intensity of the cyst fluid may decrease. Similar changes of the typical MRI appearance of cystadenoma may be caused by internal hemorrhage[26]. MRI may disclose dilated intrahepatic or extrahepatic bile ducts or demonstrate the relationship of the lesion to vascular structures and, thus, may be helpful in planning the surgical procedure.

Preoperative assumption that the lesion is benign based on US, CT or MR findings is not safe and therefore not recommended[48]. The presence of irregular thickness of the wall, mural nodules or papillary projections indicates the possibility of malignancy[2,7,45,53]. Papillary projections in the cyst can be seen on contrast-enhanced CT, and are characteristic of malignant neoplasm[4]. However, there are cases in which papillary projections are not shown clearly on CT. Hypervascularity of mural nodules on CT during arteriography may also indicate malignancy of the lesion[45]. Septation without nodularity suggests the diagnosis of cystadenoma whereas septation with mural or septal nodules, papillary infoldings, dicrete solid masses, and thick, coarse calcifications is suggestive of cystadenoc arcinoma[4,7,54]. Changes in appearance of the cyst wall may also suggest malignant transformation[45]. Despite these features, however, imaging differentiation criteria between biliary cystadenoma and cystadenocarcinoma have not yet been established[14,17,33].

Angiography is advisable and may be helpful both in clarifying the hepatic arterial anatomy and in differentiating cystadenocarcinoma from benign cystadenoma but seems not essential while the resectability of the tumor is assessed. Common findings include an avascular or hypovascular lesion with a faint rim of contrast-material accumulation in the wall and septa of the mass in the parenchymal phase and, occasionally, a tumor blush at the periphery corresponding to the papillary infoldings along with displacement of regional intrahepatic vessels[1,11,12,22,26]. Signs raising the suspicion of malignancy are considered to be attenuated intracystic vessels, stretching of thin hepatic arteries, intracystic hypervascular mural nodules,

irregular calibers of the peripheral arteries in the arterial phase, and light stains in the parenchymal phase[40,45]. Attenuated arteries and tumor stain at the central portion of an avascular or hypovascular lesion are the typical angiographic features of a cystadenocarcinoma[40].

Although difficult, correct preoperative and/or intraoperative diagnosis of cystadenomas and cystadenocarcinomas is of utmost importance in planning the appropriate surgical procedure. Among all intraoperative diagnostic and surgical procedures intraoperative ultrasound is considered the most sensitive[58]. Intraoperative US can be helpful for the diagnosis of biliary cystadenoma[3,18] and determining its resectability[11]. It may occasionally allow the surgeon to differentiate between the smooth wall of a cystadenoma and the infiltrative wall of a cystadenocarcinoma[18]. However, it cannot usually confirm the relationship between the tumor and the bile system while it may be difficult to distinguish an extrahepatic cystadenoma from a pancreatic cystadenoma because of their similar appearance on US[51]. In patients operated laparoscopically, laparoscopic ultrasonography has been reported as an essential supplement to inspection of the lesion via laparoscopy[14].

Intraoperative cholangiography is also considered very helpful since it can demonstrate both intrahepatic[12,18] and extrahepatic lesions[23]. It may allow fluid aspiration for cytology[18] and may reveal communication between the lesion and the biliary system[18] although not always[12]. In addition, it can be performed after the surgical procedure in order to exclude any bile duct injuries or retained cysts[18]. It has been proposed that intraoperative cholangiography and/or choledochoscopy should be performed to diagnose extrahepatic cystadenomas[23].

Treatment and prognosis

Resection is the management of choice for all multiloculated cystic hepatic lesions[19,59]. If a cystadenoma is suspected or has been diagnosed, surgery is indicated even in asymptomatic patients, since cystadenoma and cystadenocarcinoma cannot be reliably differentiated on the basis of radiologic and macroscopic criteria[1,5,6,10,33,48,54]. The extent of resection remains to be determined since partial resection with occasional ablation of the residual cyst using electrocautery or argon beam coagulation and/or omentopexy[14,33,60], and lobectomy[14,61] as well as wedge resection[14] and enucleation[14,19,50,62] have been reported.

In cases of communication of an intrahepatic cystadenoma with the biliary tract, biliary fistulae should be confirmed[45]. When such fistulae are identified, resection of the tumor should be supplemented with suture closure of the fistulae[14,45] or resection of the affected bile duct and bilioenteric reconstruction particularly if postoperative leak or intrahepatic biliary obstruction after suture control is concerned[14,41]. In treatment of extrahepatic cystadenomas, resection of the tumor should be supplemented with resection of the affected bile duct and bilioenteric anastomosis[17]. In cases of compression of intrahepatic or extrahepatic ducts, postoperative cholangiography may be performed to identify resolution of biliary compression[45].

Incidental finding of a cystadenoma during surgery for other clinical indications demands a complete surgical resection of the tumor[6]. Although incidental finding of a cystadenoma after open or laparoscopic fenestration of a hepatic cyst also requires complete resection, it has been proposed that after complete enucleation of the cyst, strict follow-up could be considered as the definitive treatment, demanding the surgical intervention only in case of recurrence or high suspicion of malignancy[6,63]. However, recurrence of symptoms 8 and 18 months after laparoscopic fenestration of an unsuspected cystadenoma has been reported[64].

Total tumor extirpation with a wide margin of normal liver provides a chance for cure[5,7,10,65]. Techniques other than complete excision for treatment of cystic hepatic lesions such as internal drainage, aspiration, marsupialization, sclerosis, Roux-en Y cyst-bowel anastomosis or partial resection should not be performed in cystadenomas because they may result in biliary obstruction, secondary infection or sepsis, rupture, hemorrhage, continued tumor growth, recurrence or late malignant transformation of the tumor[3,7,10,11,14,17,19,25,45,51]. Benign biliary cystadenomas are believed to transform to cystadenocarcinomas even decades after partial resection although few of these lesions have been reported[26,36,60]. Moreover, patients with hepatobiliary cystadenomas have been found to be over 10 years younger than those with cystadenocarcinomas[7]. Cystadenomas should therefore be appreciated as premalignant lesions[7,10,17,33,45,60] while cystadenoma and cystadenocarcinoma may be considered a different form of the same disease[15]. Furthermore, cystadenoma cannot be easily differentiated preoperatively or intraoperatively from cystadenocarcinoma and total surgical resection should always be considered[1,5,6,10,13,33,48,54]. Since complete surgical resection is the only safe way of eliminating such a danger and differentiating the two entities, such tumors should be completely excised[60,61].

In addition, experience with techniques such as aspiration, fenestration, internal drainage, intratumoral sclerosant application or partial resection of cystadenomas is disappointing since the recurrence rate is extremely high ranging from 90% to 100%[11,19,21,64,65] compared to 0%-10%[22,25,26,48,52,60] after radical resection. Similarly, Davies *et al*[17] reported that the recurrence rate for extrahepatic lesions is 50% after local excision from the bile duct wall compared to no recurrence after formal resection and bilioenteric reconstruction. Moreover, recurrence may be demonstrated even decades after subtotal resection[51] while consecutive recurrences in the same patient have also been reported[59].

Though still in evolution, laparoscopic surgery has an expanding role in the treatment of carefully selected patients with liver lesions and can achieve promising results[66]. There are very few reports on successful laparoscopic treatment of intrahepatic biliary cystadenomas[14,66] while no case of laparoscopically treated extrahepatic lesion has been reported. Even though laparoscopic treatment of intrahepatic cystadenomas has not been analyzed, provided that it is performed by expert liver as well as laparoscopic surgeons, the patients are carefully selected in terms of tumor size and location, and the tumor is com-

pletely excised, laparoscopic treatment is feasible and safe and may lead to similar results as open surgery[14,66].

The prognosis of patients with cystadenoma is very good if total excision of the lesion is performed[5,7,10,25,48,52]. In addition, cystadenocarcinomas may not show aggressive clinical behavior, and usually appear to have a slower growth rate and less frequent metastases or local invasion than other hepatic malignant neoplasms, such as hepatocellular carcinoma and cholangiocarcinoma, and ovarian or pancreatic cystadenocarcinomas[48]. When treated with radical excision, they also have a generally good prognosis[48,52], particularly those with mesenchymal stroma, unless the tumor invades the adjacent liver tissue or neighboring organs, or metastases are present[33,39,48].

CONCLUSION

The natural history of symptomatic progressive tumor enlargement, the inability to preoperatively differentiate cystadenoma from cystadenocarcinoma, the high possibility of recurrence after palliative procedures, and the potential for malignant transformation lend support to the thesis that biliary cystadenoma denotes a potential malignancy. Therefore total resection and a prolonged close follow-up should be performed.

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CASE REPORT

Huge primitive neuroectodermal tumor of the pancreas: Report of a case and review of the literature

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Abstract

Primitive neuroectodermal tumor (PNET) of the pancreas is an extremely rare tumor that usually occurs in children or young adults. We report a case of a 33-year-old male patient with an 18 cm × 18 cm × 16 cm mass arising from the pancreatic body and tail with a one-day history of abdominal pain. Initial CT scan showed no signs of metastatic tumor spread. The tumor caused intrabdominal bleeding and the patient underwent primary tumor resection including partial gastrectomy, left pancreatic resection and splenectomy. Diagnosis of PNET was confirmed by histology, immunohistochemistry and FISH analysis. All neoplastic cells were stained positive for MIC2-protein (CD99). Approximately one month after surgery, several liver metastases were observed and the patient underwent chemotherapy according to the Euro-Ewing protocol. Subsequent relaparotomy excluded any residual hepatic or extrahepatic abdominal metastases. Although PNET in the pancreas is an extremely rare entity, it should be considered in the diffential diagnosis of pancreatic masses, especially in young patients. This alarming case particularly illustrates that PNET in the pancreas although in an advanced stage can present with only a short history of mild symptoms.

Key words: Primitive neuroectodermal tumor; Pancreas; MIC2-protein; Ewing sarcoma; Abdominal mass

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INTRODUCTION

Primitive neuroectodermal tumors (PNETs) are small round cell tumors arising from soft tissue belonging to the Ewing's sarcoma family. These neoplasms all exhibit a neural phenotype, express the MIC2-protein (CD99) and display the same chromosomal translocation t (11; 22) (q24; q12) in about 85% of the cases[1]. While Ewing sarcoma is a primary bone tumor and follows osteosarcoma as the second most common malignant bone tumor in children, PNETs occur most often in soft tissue of the thoracopulmonary region, pelvis and lower extremities of children and young adults. Most of these tumors are diagnosed before the age of 35 years with a slight predominance in male patients[1]. Although PNETs can occur in numerous solid organs such as the kidney, ovary, vagina, testis, uterus, cervix uteri, urinary bladder, parotid gland, heart, lung, rectum and pancreas, it is an extremely rare tumor entity[2]. According to the literature there are currently 11 reported cases of PNET originating from the pancreas (Table 1)[2-6].

Here we report another case of PNET in the pancreas in an otherwise healthy 33-year old man.

CASE REPORT

A 33-year old man presented to the Emergency Department of our hospital with acute abdominal pain which started one day ago. The patient reported one episode of vomiting but no history of diarrhea, peptic ulcer disease or pancreatitis. Laboratory data on admission showed leucocytosis, elevated C-reactive protein, amylase and lipase.

CT-scan of the abdomen revealed a 13.7 cm × 15.0 cm mass arising from the pancreatic tail compressing the stomach and spleen (Figure 1). The mass had solid and cystic characteristics with an inhomogenous contrast enhancement. Furthermore, blood was noted in the subhepatic area and pelvis indicating rupture of the tumor. Numerous mesenteric lymph nodes were enlarged up to 11 mm, while the liver showed no lesions suspicious of metastasis. Until surgery, a significant decline in hematocrit due to tumor bleeding was observed. At

Table 1 Overview of reported cases of pancreatic PNET

Reference	Age (yr)	Gender	Tumor origin	Tumor size (cm)	Therapy	Survival (cause)
Lüttges *et al*[5] 1997	13	Female	Pancreatic body/tail	22 × 8 × 10	Resection, chemotherapy	NR
	31	Male	Pancreatic body	-	Chemotherapy, resection	NR
Bulchmann *et al*[3] 2000	6	Female	Pancreatic head	4.0 × 5.4 × 3.0	Whipple resection with colon segmentectomy	6 mo (RD)
Movahedi-Lankarani *et al*[2] 2002	6-25 (mean 18)	4 male, 3 female	Pancreatic head	3.5 to 9.0	Whipple resection in 4 cases (2 VDC), biopsy in 3 cases (1 VDC)	Up to 48 mo
Perek *et al*[6] 2003	31	Male	Pancreatic head and body	10 × 12	1 Whipple resection (radiochemotheray denied), 2 Local resection + chemotherapy, 3 Resection of lung metastasis + chemotherapy	50 mo (RD)
Present case	33	Male	Pancreatic body	18 × 18 × 16	Resection, 6 cycles VIDE, VAI, AST	Alive at 1 year after diagnosis

AST: Autologous stem cell transplantation; mo: Months; NR: Not reported; RD: Recurrent disease; VDC: Vincristin, doxorubicin, cyclophosphamide; VIDE: Vincristin, ifosfamide, doxorubicin and etoposide; VAI: Vincristin, actinomycin D: Ifosfamide.

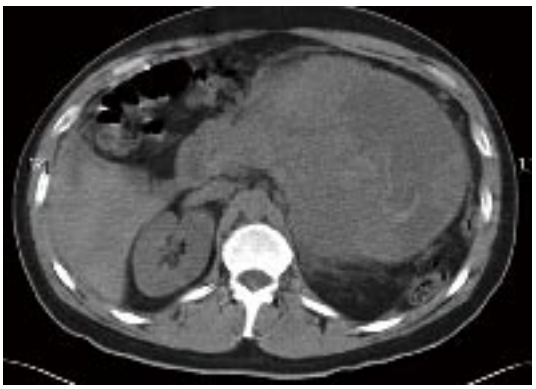

Figure 1 CT-scan showing a huge intra-abdominal mass with solid and cystic areas originating from the pancreas.

laparotomy the tumor was found to be adherent to the stomach and the pancreatic body and tail. The capsule was partially ruptured. An enbloc-resection of the tumor was accomplished by partial gastric resection (Billroth-Ⅱ), left pancreatic resection and splenectomy. The continuity was restored with gastrojejunostomy and Roux-Y-jejunojejunostomy. The postoperative course was uneventful.

Grossly, the tumor measuring 18 cm × 18 cm × 16 cm was surrounded by an edematous capsule and appeared as cystic with alternating necrotic areas. The tumor was adhered to the pancreatic tail and stomach adjacent to the minor curvature where two small non-neoplastic ulcers were noted.

On microscopic examination, the solid tumor parts were composed of nests of medium-sized round or oval tumor cells with enlarged round or oval nuclei and scant cytoplasm surrounded by fibrovascular septae (Figure 2A). Focally, Homer-Wright rosettes were observed (Figure 2A). Immunohistochemically, the tumor showed a consistent and strong membraneous expression of CD99 (Figure 2B) and a strong cytoplasmic staining for vimentin (Figure 2C). NSE-positive and negative neoplastic cells were found in about equal proportions, and smaller neoplastic subsets were positive for cytokeratin (KL-1; Figure 2D), cytokeratin 18, EMA, synaptophysin, CD56 (Figure 2E) and CD117. The neoplastic cells lacked any detectable cytokeratins (7, 8 and 19), CEA, α1-fetoprotein (AFP), α1-antichymotrypsin (α1ACT), protein S100, melan A and HMB-45. The Ki67 (MIB-1) labeling index was about 20%-30% (Figure 2F). Fluorescence *in situ*-hybridization was performed with a dual-colour DNA probe flanking the EWS gene on chromosome 22q12 (Vysis/Abbott, Wiesbaden, Germany) according to the manufacturer's instructions. Tumour cell nuclei showed one fused signal and one dislocated hybridization signal indicative of a chromosomal translocation involving the EWS gene. Overall, the microscopic findings led to the diagnosis of PNET.

Following primary hospitalization and surgery the patient was scheduled for staging examinations prior to chemotherapy. Thirty-five days after laparotomy, CT and MRI revealed at least two liver metastases in segments 6 and 7 up to 1.5 cm in size that were biopsied and confirmed immunohistochemically. There were no metastases in the lung, bone marrow or in the skeletal system according to staging by CT, bone marrow biopsy and scintigraphy. The patient underwent 6 cycles of induction VIDE chemotherapy (vincristin, ifosfamide, doxorubicin and etoposide), 1 cycle of VAI chemotherapy (vincristin, actinomycin D, ifosfamide) followed by high dose chemotherapy with melphalan and etoposide and autologous stem cell transplantation. During chemotherapy, devitalization and shrinking of the liver metastases were documented. Because CT visualized a residual lesion in segment 6 of the liver, the patient underwent explorative laparotomy with intraoperative ultrasound which did not show any metastasis or residual tumor. One year after diagnosis, there was no evidence of tumor recurrence.

DISCUSSION

Ewing sarcoma (EWS) and PNET belong to a tumor family (Ewing family of tumors) that is characterized

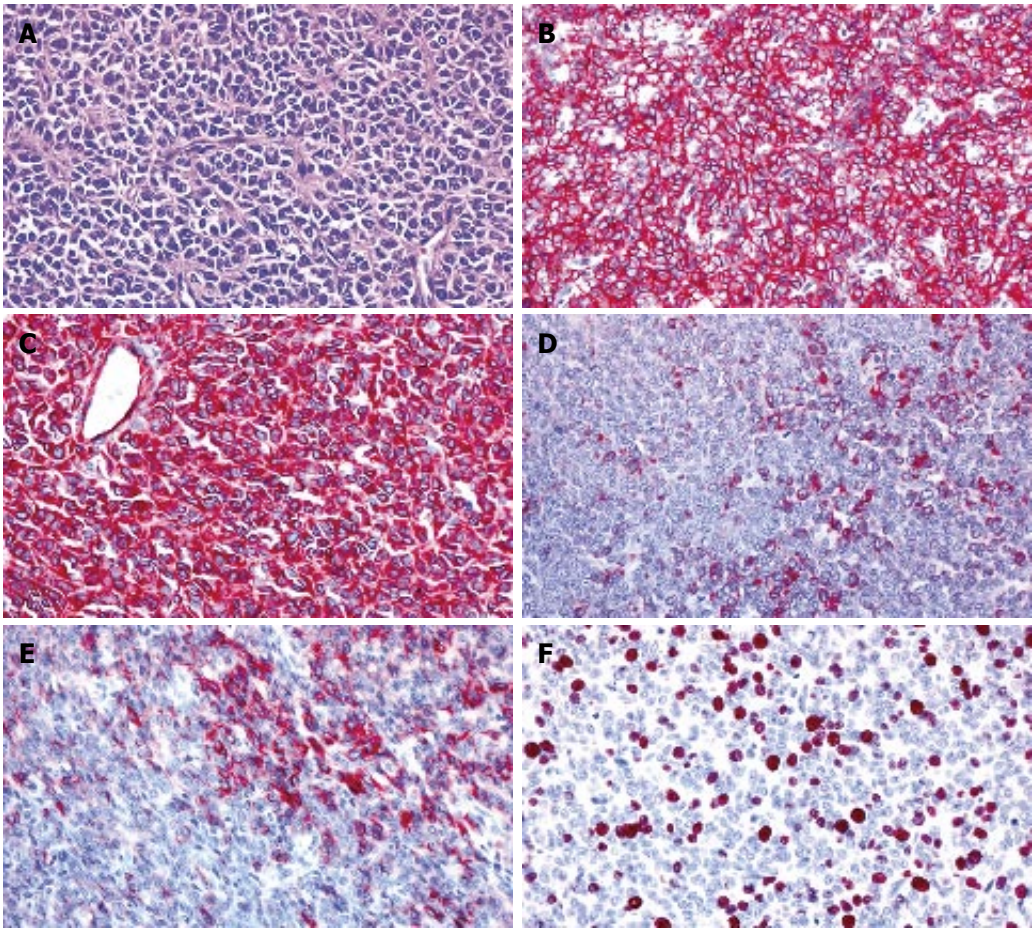

Figure 2 Histomorphological characteristics and immunolabeling of PNET. **A**: Solid tumor cell sheets separated by a delicate fibrovascular stroma; **B**: Strong membranous expression of CD99 (MIC-2) in the entire tumor cell population; **C**: Vimentin-positive neoplastic cells; **D**: Expression of cytokeratins (KL-1) in a minor tumor cell subset; **E**: Partial CD56-positive neoplastic population; **F**: Nuclear staining for the Ki67 proliferation antigen (MIB-1) in about 20%-30% of tumor cells.

by typical chromosomal translocations with subsequent functional fusion of the EWS gene with transcription factor genes thus forming a chimeric protein. Peripheral PNET (pPNET) was first described by Stout[7] as a tumor of the ulnar nerve with the gross features of a sarcoma but composed of small round cells focally arranged as rosettes in 1918. pPNET makes up approximately 1% of all sarcomas[2]. EWS and PNET form a subset of the "small-round-cell tumors" in childhood that comprise lymphomas, neuroblastoma and soft tissue sarcomas including rhabdomyosarcomas as well, which at least in undifferentiated forms, are indistinguishable by conventional light microscopy. When PNET is found in the pancreas, the differential diagnosis includes undifferentiated small cell carcinoma, pancreatoblastoma and pancreatic endocrine tumors. Thus the diagnosis of PNET necessitates histopathologic, immunohistochemical and if possible, also genetic analysis. EWS and PNET show a high expression of the cell surface glycoprotein MIC2 (also named CD99 or $p30/32^{MIC2}$) which is considered to be important in cell adhesion[1]. However, other neoplasms as well as normal tissue including pancreatic endocrine tumors and pancreatic islet cells express the MIC2 protein, thus limiting specificity of the test.

Cytogenetic and molecular analysis of translocations has been established as a powerful adjunct for sarcoma classification. EWS/PNET show typical chromosomal translocations involving the EWS gene on chromosome 22 and a member of the ETS family of genes which code for DNA-binding transcription factors. The most common translocation *t* (11; 22) (q24; q12) resulting in the fusion product EWS-FLI1 occurs in 85%-95 %, while the second most common translocation *t* (21; 22) (q22; q12) is seen in 5%-10%[1].

Once PNET is diagnosed, the standard treatment is a systemic multi-agent chemotherapy combined with surgery and/or radiotherapy. Tumor dissemination at the time of diagnosis is associated with a poorer outcome compared to localized disease[8]. However, a retrospective study on 24 patients younger than 16 years old with extraskeletal EWS reported that age and surgical treatment, but not size of tumor and metastatic disease at the time of diagnosis are significant prognostic factors[9]. In this study the overall 5-year survival rate was 61% and the disease free survival rate was 54%. In various series including patients with extraskeletal EWS, the 5-year survival rate ranges from 61% to 77%[8]. Intensive preoperative multi-agent chemotherapy may further improve the prognosis of EWS family tumors[10]. There are only few data focusing on the prognosis of patients with PNET in the pancreas. Movahedi-Lankarani *et al*[2] reported 2 patients with chemotherapy after primary Whipple procedure. One was alive at 33 mo with no evidence of disease, while the other died of disease 4 years after diagnosis.

In conclusion, PNET of the pancreas is an extremely rare pancreatic tumor and mainly affects children or young adults. To our knowledge, this is the 12th-reported case so

far. Although uncommon, PNET has to be considered in the differential diagnosis of atypical pancreatic tumors in young patients.

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CASE REPORT

Intracolonic multiple pebbles in young adults: Radiographic imaging and conventional approach to a case

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Abstract

Most of the foreign bodies detected in adult gastrointestinal systems are accidentally swallowed pins. In this study, we presented a case with intracolonic multiple pebbles. A 20-year-old man was admitted to emergency surgery policlinic for abdominal pain for 2 d without any alleviation or aggravation. His upright plain abdominal radiographic imaging revealed about 30-40 overt dense opacities in lumen of colonic segments, with oval and well shaped contours, each approximately 1 cm x 1 cm in size. The multiplanar reconstructions and three-dimensional images combined with sectional screening showed that all pebbles had passed completely into the colon and no foreign bodies had remained in the ileal segments. On psychiatric assessment, he was found to have immature personality features, difficulty in overcoming stressors and adaptation disorder. He recovered by conservative management and radiographic monitoring applied during his follow-up. Thus, it can be concluded that, in differential diagnosis of abdominal pain in adult ages, though less frequently seen than in children, gastrointestinal system foreign bodies should always be kept in mind and it should be considered that ingestion of pebbles may be one of the factors contributing to abdominal pain particularly in young adults with psychiatric problems. In such cases suspected of having foreign bodies which cannot be detected by plain films, abdominal tomography can be an alternative for diagnostic imaging.

Key words: Intracolonic multiple pebbles; Current approach; Radiographic imaging

Eryilmaz M, Ozturk O, Mentes O, Soylu K, Durusu M, Oner K. Intracolonic multiple pebbles in young adults: Radiographic imaging and conventional approach to a case. *World J Gastroenterol* 2006; 12(37): 6074-6076

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INTRODUCTION

The frequency of foreign body detection in gastrointestinal system is less in adulthood compared to childhood. Accidentally swallowed pins account for most of the foreign bodies found in adult gastrointestinal systems. Oral dental implants, bezoars, chicken-fish bones, packages of medications and drugs are among the other foreign bodies commonly seen to be swallowed[1]. Incidence of foreign bodies swallowed by an individual with mental defect or due to conversive reaction is relatively rarer. Regardless of the types of the objects, foreign bodies are very rare to result in a serious clinical condition in intestinal lumen. If it does not develop an emergency surgical indication, there is no need to hospitalize the cases. Conservative follow-up approach is a generally preferred method. Out-patient follow-up through serial physical examination and plain films will be enough. A good observation and quick radiographic monitoring is suggested for the follow-up of such patients. As long as the objects do not get stuck in intestinal transition points such as pylorus or ileo-caecal valve, they are generally passed rectally out within a few days[2]. We present herein a young adult case of intracolonic multiple pebbles, who was managed successfully by serial radiographic imaging and conventional approach, without surgical intervention.

CASE REPORT

A 20-year-old male presented in a peripheral hospital with the complaint of abdominal pain. With a provisional diagnosis of "intestinal foreign body", based on his upright abdominal plain film, he was referred to our hospital. The abdominal pain had been continuing for 2 d, with mild severity without any alleviation or aggravation. His physical examination revealed soft abdomen, no guarding and rebound tenderness, and normal bowel sound. However, tenderness was found at right lower quadrant on deep palpation. No abnormality was detected in rectal examination. Complete blood count and urine test were within normal limits. His upright abdominal plain film showed about 30-40 overt dense opacities in the colonic segments, with oval and well shaped contours, each approximately 1 cm × 1 cm in size (Figure 1). In addition to these multi-opacities, an image of a pin was detected in the region consistent

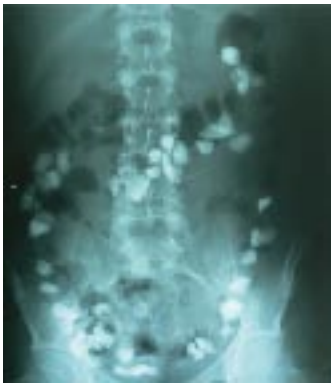

Figure 1 An upright plain abdominal radiographic imaging revealing 30-40 overt dense opacities in lumen of colonic segments, with oval and well shaped contours, each approximately 1 cm x 1 cm in size.

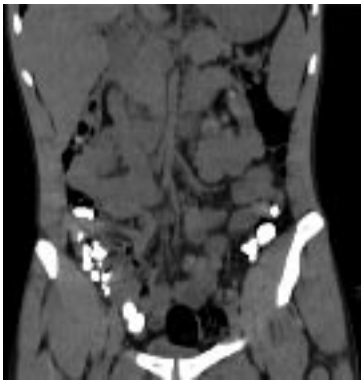

Figure 2 CT showing dense opacities in lumen of colonic segments, with oval and well shaped contours.

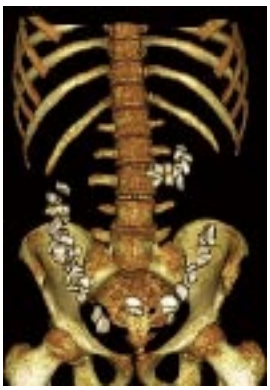

Figure 3 A multiplanar reconstruction and three-dimensional image combined with sectional screening showing all pebbles had passed completely into the colon and no foreign bodies were remained in the ileal segments.

with the right lower quadrant. In abdominal ultrasound, there were not any obvious abnormalities supporting the findings of the plain film. However, a minimal amount of smear-like fluid was present around the ileal segments in right lower quadrant. Low-dose computed abdominal tomography was applied without administering contrasting substance to see whether there is a foreign body which is unable to pass into the colon and poses the risk of obstruction, to be able to detect other foreign bodies which might not show any opacity, to evaluate the ileo-caecal valve better, and to better analyze the smear-like fluid shown by ultrasonography. In the lumen of colon, about 30-40 hyperdense images, suspicious of being pebbles, were detected (Figure 2). The multiplanar reconstructions and three-dimensional images combined with sectional screening revealed that all pebbles had passed completely into the colon and no foreign bodies had remained in the ileal segments (Figure 3). After computed abdominal tomography, rectal enema was applied to the patient and a few pebbles were passed out the body through defecation. He was managed conservatively and monitored with physical examination and abdominal plain film on follow-up in surgical out-patient clinic, until all the pebbles and pin were discharged within one week *via* defecation without any difficulty.

DISCUSSION

Intestinal foreign body cases resulting from foreign body ingestion accidentally or due to a conversive reaction are seen less frequently in adulthood when compared to childhood. Types of the intestinal foreign bodies taken orally by adults differ from those taken by children. In literature, plastic pipe, metal pin, nail, screw, spoon, bamboo stick and paper clip have been reported in adults as gastrointestinal system foreign bodies[3-5]. Some case reports are available regarding Meckel's diverticulum perforations caused by swallowed fish bone[6-8], foreign bodies[9-11], chicken bone[12] and alkaline battery[13]. In the relevant literature, Selivanov *et al*[14] published a series of 101 and Steven *et al*[15] a series of 75 foreign body ingestion cases. In addition, Losanoff *et al*[5] presented a series of 9 prisoners who swallowed metal parts shaped like a cross.

Orally ingested foreign bodies are the cases that are not seen frequently in emergency surgery policlinic. The basic approach is physical examination and imaging techniques. Patients considered not having acute abdominal syndrome after physical examinations are followed up with a serial upright abdominal plain film and physical examination. In case the foreign body cannot be visualized in conventional films, it should be kept in mind that the patient did not swallow the object or conventional techniques remained inadequate to visualize it. When the presence of other foreign bodies is suspected although one is observed on plain films, computed abdominal tomography can be considered as a proper alternative[16-20]. Plain film findings of our case were evaluated in accordance with literature. Abdominal ultrasonography was applied considering that there might be foreign bodies which cannot be seen on plain films. Pebbles and the pin detected on plain films could not be seen on ultrasonography. Then computed abdominal tomography was applied to the case, which revealed multiple opacities that were suspected of being pebbles with intracolonic localization.

Intestinal foreign body cases, regardless of being in adulthood or childhood, do not reveal any symptoms as long as the object does not cause obstruction. If they lead to any obstruction or perforation, the main symptom to emerge will be abdominal pain, as was found in our case. Data have shown that majority of the cases are normal adults who swallowed fish or chicken bones while eating or mentally retarded patients who swallowed foreign bodies unconsciously.

His physical examination revealed soft abdomen, no guarding and rebound tenderness, and normal bowel sound. However, tenderness was found at right lower quadrant on deep palpation. No acute abdominal symptom or a clinical condition requiring an emergency surgi-

cal intervention could not be obtained in the case. The swallowed foreign bodies were clearly seen on his upright abdominal plain film. Literature points out that metal and glass objects can be seen on plain films, but not wooden objects, so abdominal USG may be obtained when deemed necessary[21]. It will be very helpful to evaluate the abdomen through computed tomography in addition to plain films particularly in cases with psychiatric problems, suspicious of swallowing objects such as drugs, posing the risk of harming themselves and in those trying to conceal the truth, as seen in our case. Computed tomography and three-dimensional imaging were applied to the case in order to reveal other foreign bodies that might be present although they could not be monitored on plain films and to eliminate the foreign bodies that might remain in ileal segments and lead to ileo-caecal obstruction. Since there might be some other foreign bodies that cannot be detected by plain films, computed tomography and three-dimensional imaging are applied as the appropriate alternative for the detailed assessment of the intestinal lumen in suspected problematic cases and in the elimination of probable future complications.

While approaching to the intestinal foreign body cases, location of the foreign body has a great importance in terms of management. According to the localization and shape of the object, it is decided on whether an out-patient treatment or surgical intervention will be applied[17,20,21]. Although sharp objects pass through gastrointestinal tract without difficulty in 90% of the cases, they may also be removed using fiberoptic gastroscopy without waiting. Early endoscopic intervention, in general, may be required and applied for objects wider than 2 cm and longer than 6 cm, since their passage through pylorus and duodenum will become difficult. Once these foreign bodies pass beyond pylorus and ileo-caecal valve, they are easily removed out of the lumen[2]. Losanof *et al*[12] operated all cases since the foreign bodies have pointed and sharp edges. Our case was managed conservatively and monitored on out-patient policlinic follow-up, in the light of the literature. He was informed about the probable progress of his condition and suggested to be admitted to the clinic as soon as possible in case of acute abdominal pain and/or any other complaints.

In conclusion, in differential diagnosis of abdominal pain seen in adult ages, gastrointestinal system foreign bodies should always be kept in mind although they are seen less frequently than in children. It should be considered that intestinal system foreign bodies may be one of the factors contributing to abdominal pain particularly in young adults. In such cases suspected of having foreign bodies which cannot be detected by plain films, abdominal tomography can be a sound alternative for diagnostic imaging.

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CASE REPORT

Sigmoid colonic carcinoma associated with deposited ova of *Schistosoma japonicum*: A case report

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Abstract

We report a case of sigmoid colonic carcinoma associated with deposited ova of *Schistosoma japonicum*. A 57-year old woman presented with a 10-mo history of left lower quadrant abdominal pain and a 2-mo history of bloody stools. She had a significant past medical history of asymptomatic schistosomiasis japonica and constipation. A colonoscopy showed an exophytic fragile neoplasm with an ulcerating surface in the sigmoid colon. During the radical operative procedure, we noted the partially encircling tumor was located in the distal sigmoid colon, and extended into the serosa. Succedent pathological analysis demonstrated the diagnosis of sigmoid colonic ulcerative tubular adenocarcinoma, and showed deposited ova of *Schistosoma japonicum* in both tumor lesions and mesenteric lymph nodes. Three days after surgery the patient returned to the normal bowel function with one defecation per day. These findings reveal that deposited schistosome ova play a possible role in the carcinogenesis of colorectal cancer.

Key words: Schistosomiasis japonica; Sigmoid colonic carcinoma; Deposited schistosome ova

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INTRODUCTION

Schistosomiasis is a trematode parasitic infection in which terminal hosts are humans or other mammals with freshwater snails as intermediate hosts[1]. Schistosomiasis japonica is endemic mainly in China, and remains a major public health problem today although remarkable successes in schistosomiasis control have been achieved over the previous four decades[2].

The life cycle of *Schistosoma japonicum (S. japonicum)* starts when cercariae in the freshwater infect humans or other mammals. After maturing, male and female worms pair up in the hepatic portal vein system, and produce ova in the mucosal branches of the inferior mesenteric and superior hemorrhoidal veins. Many ova are discharged in the feces, hatch, and release free-swimming miracidia, which in turn reinfect receptive freshwater snails and asexually produce larval cercariae[2]. However, some ova deposit in the tissues of the host, cause host inflammatory responses, and lead to granuloma formation which is the principal pathology associated with schistosomiasis[3]. Periovular granulomas have been found in many types of tissue, including the liver, intestine, skin, lung, brain, adrenal glands, and skeletal muscle. Ova retained in the gut wall induce inflammation, hyperplasia, ulceration, microabscess formation, polyposis, and carcinogenesis[3].

Successes in schistosomiasis control in China have prolonged the life of patients with schistosomiasis japonica in which the chronic pathological process may occur due to deposited schistosome ova.

Here we report a case of sigmoid colonic carcinoma associated with deposited ova of *S. japonicum*, with their possible role in carcinogenesis discussed.

CASE REPORT

A 57-year old woman presented with a 10-mo history of left lower quadrant abdominal pain and a 2-mo history of bloody stools. She had a sense of pain in the left lower quadrant abdomen when she had bowel movements about 10 mo ago. The pain was relieved after defecation, so that she did not think much of it. Two months ago, she noted that dark red blood adhered to the surface of stools without blood drops from anus. The symptom of hematochezia persisted until hospitalization. She had no other complaints. She had a significant past medical history of asymptomatic schistosomiasis japonica and constipation. She came from the rural area in Zhejiang Province, where schistosomiasis japonica was prevalent several decades ago. She had the experience of swimming in a local river in her childhood. At the age of 13, she was diagnosed with schistosomiasis through a thick smear stool examination by the county public health workers

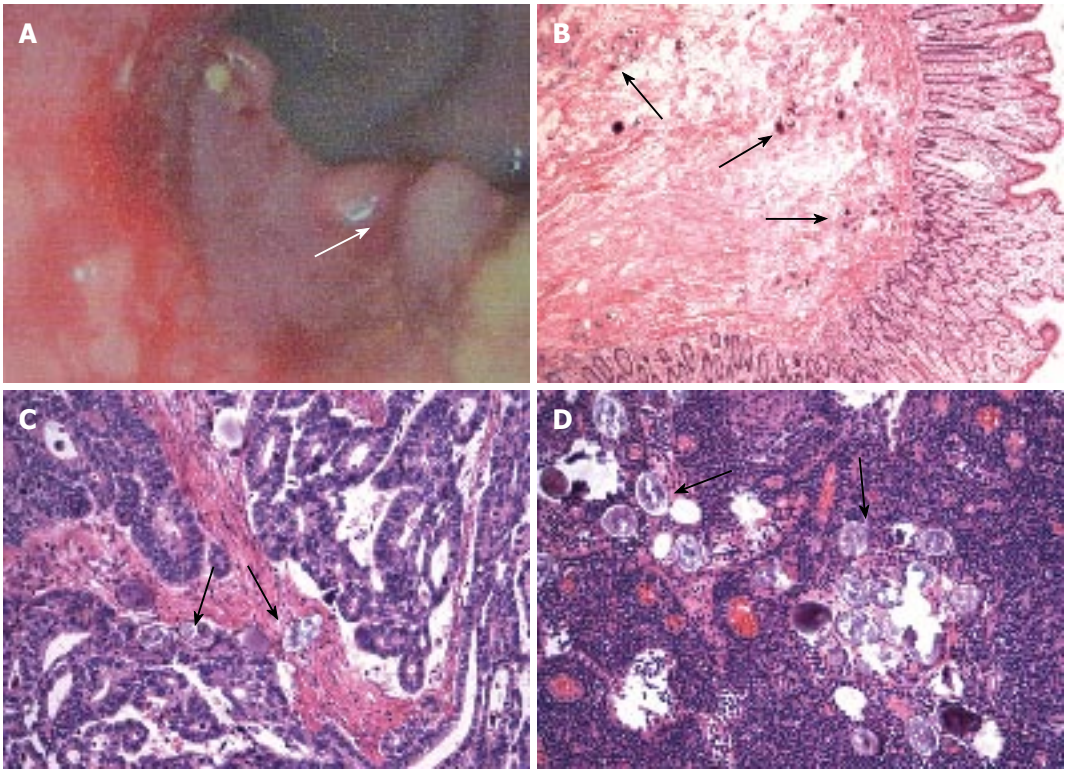

Figure 1 Colonoscopy showing an exophytic fragile neoplasm with an ulcerating surface (as indicated by the white arrow head) in the sigmoid colon (**A**), pathological analysis revealing deposited *S. japonicum* ova and granuloma formation as well as fibrotic deposition in the submucosa of the sigmoid colon (**B**) (HE × 50), moderately-differentiated tubular adenocarcinoma (HE × 200) and deposited *S. japonicum* ova in the tumor (**C**), and deposited *S. japonicum* ova in the mesenteric lymph node (**D**) (HE × 200). Black arrow heads indicate *S. japonicum* ova.

for schistosomiasis control, and given the appropriate antischistosomiasis therapy which was not terminated until repeated stool examinations were negative. At the age of 20, she suffered from constipation which was not improved by adequate fiber intake. Usually, she had about two bowel movements with hard stools per week. The symptom began to become worse ten years ago and she had defecation once per week.

She was in good general condition. No supraclavicular lymph nodes were palpable. On abdominal palpation, there was a discomfortable sense in the left lower quadrant without palpable masses. Her peripheral blood cell count showed leucopenia (white blood cells: 3100/mm^3) and mild thrombocytopenia (platelets: 72000/mm^3), without anemia and eosinophilia. Three stool tests were all negative for *S. japonicum* ova. Liver function, renal function and electrocyte concentration were all normal. Tumor markers (AFP, CEA, and CA-199) were 2.41 ng/mL, 0.72 ng/mL, and 5.88 U/mL, respectively, which were within normal limits. The abdominal ultrasonography revealed echodense areas scattered within the parenchyma with typical fish-scale pattern, representing the trait of liver schistosomiasis. The computed tomographic scan showed that the bowel wall of sigmoid colon was incrassated and irregular in shape. The colonoscopy showed an exophytic fragile neoplasm with an ulcerating surface in the sigmoid colon (Figure 1A), which was diagnosed as adenocarcinoma through biopsy.

After definite diagnosis was made, the radical operative procedure was performed. During laparotomy, the entire peritoneal cavity was examined, with a thorough inspection of the liver, pelvis, and hemidiaphragm. Neither metastatic lesions nor enlarged mesenteric lymph nodes were found. The partially encircling tumor was located in the distal sigmoid colon, and extended into the serosa. Pathological analysis of the operative specimen revealed deposited *S. japonicum* ova, granuloma formation, and fibrotic deposition in the submucosa of the sigmoid colon (Figure 1B), and demonstrated a grade 2 moderately-differentiated sigmoid colonic ulcerative tubular adenocarcinoma, 3 cm × 2 cm in size, penetrating the serosa without lymph node involvement (Figure 1C). Deposited *S. japonicum* ova were found in both tumor lesions (Figure 1C) and mesenteric lymph nodes (Figure 1D).

Three days after surgery the patient returned to the normal bowel function with one defecation per day.

DISCUSSION

The relation between colorectal cancer and schistosomiasis japonica has not been well established. However, researchers in China have noted the coincidence of colon cancer and schistosomiasis japonica at the patient level during the 1970s[4]. Another case-control study in Jiangsu Province showed that rectal cancer is associated with schistosomiasis japonica[5]. A recent matched case-control study showed that previous *S. japonicum* infection is significantly associated with both liver cancer and colon cancer[6]. It was also reported that schistosomiasis japonica correlates with colorectal cancer [7].

The mechanism of schistosome infection-induced colorectal cancer was explored in our study. The mutations in p53 tumor suppressor gene in Chinese patients with schistosomiasis japonica-related rectal cancer and a high proportion of base-pair substitutions at CpG dinucleotides and a higher frequency of arginine missense mutations were observed, suggesting that the mutations are the result of genotoxic agents produced endogenously

through the course of schistosomiasis japonica[8]. Another study showed that *S. japonicum* ova-induced colorectal epithelial proliferative polyps have a high percentage of atypical hyperplasia (64.9%) and CEA (90%)[9]. Thus the epithelial proliferative polyp, especially that with atypical hyperplasia, is the transition during deposited ova-induced carcinogenesis. It was reported that the majority of colorectal cancer cases associated with *Schistosoma mansoni* (*S. mansoni*) infection have a significant expression of Bcl-2 while p53 and C-Myc expressions are insignificantly different in colorectal cancer cases associated with *S. mansoni* infection compared with those without *S. mansoni* infection[10]. In a word, the mechanism of schistosome infection-induced carcinogenesis remains unclear.

In our present case, both chronic constipation and sigmoid colonic adenocarcinoma were induced by deposited *S. japonicum* ova, revealing that ova can interact with the host microenvironment. Asymptomatic schistosomiasis in our patient was cured as neither eosinophilia nor ova in her stools were observed. However, many old ova deposited in sigmoid colonic submucosa, tumor lesions, and mesenteric lymph nodes. Furthermore, the anatomic location of adenocarcinoma corresponded to the colorectal area with most deposited ova. These findings indicate that deposited *S. japonicum* ova play a possible role in the carcinogenesis of colorectal cancer[7].

Another important aspect of this case is that chronic constipation was improved after operation. In this case, granuloma formation and fibrotic deposition around deposited ova perhaps disturbed the sensory function of the sigmoid colon, and resulted in constipation.

Additionally, we noted deposited ova in the mesenteric lymph nodes, leucopenia, and thrombocytopenia in this case. However, their pathological significance remains unclear.

In conclusion, sigmoid colonic carcinoma is associated with deposited ova of *S. japonicum*. Therefore, screening for colorectal cancer should always be thoroughly performed with routine endoscopy in patients with previous *S. japonicum* infection, and the mechanism of deposited *S. japonicum* ova-induced carcinogenesis should be further investigated.

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Meetings

MAJOR MEETINGS COMING UP

First Biennial Congress of the Asian-Pacific Hepato-Pancreato-Biliary Association
March, 2007
Fukuoka, Japan
http://www.congre.co.jp/1st-aphpba

American College of Gastroenterology Annual Scientific
20-25 October 2006
Las Vegas, NV

14th United European Gastroenterology Week, UEGW
21-25 October 2006
Berlin, Germany

APDW 2006: Asian Pacific Digestive Week 2006
26-29 November 2006
Lahug Cebu City, Philippines

EVENTS AND MEETINGS IN THE UPCOMING 6 MONTHS

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24-25 March 2006
Sydney - NSW
Falk Foundation e.V.
symposia@falkfoundation.de

10th International Congress of Obesity
3-8 September 2006
Sydney
Event Planners Australia
enquiries@ico2006.com
www.ico2006.com

Easl 2006 - the 41st annual
26-30 April 2006
Vienna, Austria
Kenes International

Prague hepatology 2006
14-16 September 2006
Prague
Foundation of the Czech Society of Hepatology
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

12th International Symposium on Viral Hepatitis and Liver Disease
1-5 July 2006
Paris
MCI France
isvhld2006@mci-group.com
www.isvhld2006.com

Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4-5 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6-7 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

ILTS 12th Annual International Congress
3-6 May 2006
Milan
ILTS
www.ilts.org

Internal Medicine: Gastroenterology
22 July 2006-1 August 2006
Amsterdam
Continuing Education Inc
jbarnhart@continuingeducation.net

6th Annual Gastroenterology And Hepatology
15-18 March 2006
Rio Grande
Office of Continuing Medical Education
cmenet@jhmi.edu
www.hopkinscme.net

World Congress on Gastrointestinal Cancer
28 June 2006-1 July 2006
Barcelona, Spain
c.chase@imedex.com

International Conference on Surgical Infections, ICSI2006
6-8 September 2006
Stockholm
European Society of Clinical Microbiology and Infectious Diseases
icsi2006@stocon.se
www.icsi2006.se/9/23312.asp

7th World Congress of the International Hepato-Pancreato-Biliary Association
3-7 September 2006
Edinburgh
Edinburgh Convention Bureau
convention@edinburgh.org
www.edinburgh.org/conference

Society of American Gastrointestinal Endoscopic Surgeons
26-29 April 2006
Dallas - TX
www.sages.org

Digestive Disease Week 2006
20-25 May 2006
Los Angeles
www.ddw.org

Annual Postgraduate Course
25-26 May 2006
Los Angeles, CA
American Society of Gastrointestinal Endoscopy
www.asge.org/education

American Society of Colon and Rectal Surgeons
3-7 June 2006
Seatlle - Washington
www.fascrs.org

EVENTS AND MEETINGS IN 2006

10th World Congress of the International Society for Diseases of the Esophagus
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isde@sapmea.asn.au
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In press

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No author given

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